



IMPROVED HEAT SHOCK PROTEIN-BASED VACCINES AND IMMUNOTHERAPIES

CROSS-REFERENCE TO RELATED APPLICATIONS

5 [0001] This application claims priority under 35 U.S.C. § 119(e) to provisional applications 60/447,142, filed February 13, 2003; 60/462,469, filed April 11, 2003; 60/463,746, filed April 18, 2003; and 60/503,417, filed September 16, 2003, all four of which are incorporated herein by reference in their entireties.

INTRODUCTION

10 [0002] The present invention relates to methods and compositions for inducing an immune response in a subject, wherein the subject is administered an effective amount of at least one or more defined hybrid antigens optionally in combination with one or more heat shock proteins. These methods and compositions may be used in the treatment of infectious diseases and cancers.

BACKGROUND OF THE INVENTION

15 [0003] Heat shock proteins were originally observed to be expressed in increased amounts in mammalian cells which were exposed to sudden elevations of temperature, while the expression of most cellular proteins is significantly reduced. It has since been determined that such proteins are produced in response to various types of stress, including glucose deprivation. As used herein, the term "heat shock protein" will be used to
20 encompass both proteins that are expressly labeled as such as well as other stress proteins, including homologues of such proteins that are expressed constitutively (i.e., in the absence of stressful conditions). Examples of heat shock proteins include BiP (also referred to as grp78), hsp70, hsc70, gp96 (grp94), hsp60, hsp40 and hsp90.

25 [0004] Heat shock proteins have the ability to bind other proteins in their non-native states, and in particular to bind nascent peptides emerging from ribosomes or extruded into the endoplasmic reticulum. Hendrick and Hartl, *Ann. Rev. Biochem.* 62:349-384 (1993); Hartl, *Nature* 381:571-580 (1996). Further, heat shock proteins have been shown to play an important role in the proper folding and assembly of proteins in the cytosol, endoplasmic

reticulum and mitochondria; in view of this function, they are referred to as "molecular chaperones." Frydman et al., *Nature* 370:111-117 (1994); Hendrick and Hartl, *Ann. Rev. Biochem.* 62:349-384 (1993); Hartl, *Nature* 381:571-580 (1996).

[0005] For example, the protein BiP, a member of a class of heat shock proteins referred to as the hsp70 family, has been found to bind to newly synthesized, unfolded μ immunoglobulin heavy chain prior to its assembly with light chain in the endoplasmic reticulum. Hendershot et al., *J. Cell Biol.* 104:761-767 (1987). Another heat shock protein, gp96, is a member of the hsp90 family of stress proteins which localizes in the endoplasmic reticulum. Li and Srivastava, *EMBO J.* 12:3143-3151 (1993); Mazzarella and Green, *J. Biol. Chem.* 262:8875-8883 (1987). It has been proposed that gp96 may assist in the assembly of multi-subunit proteins in the endoplasmic reticulum. Wiech et al., *Nature* 358:169-170 (1992).

[0006] It has been observed that heat shock proteins prepared from tumors in experimental animals were able to induce immune responses in a tumor-specific manner; that is to say, heat shock protein purified from a particular tumor could induce an immune response in an experimental animal which would inhibit the growth of the same tumor, but not other tumors. Srivastava and Maki, *Curr. Topics Microbiol.* 167:109-123 (1991). Genes encoding heat shock proteins have not been found to exhibit tumor-specific DNA polymorphism. Srivastava and Udonon, *Curr. Opin. Immunol.* 6:728-732 (1994). High resolution gel electrophoresis has indicated that gp96 may be heterogeneous at the molecular level. Feldweg and Srivastava, *Int. J. Cancer* 63: 310-314 (1995). Evidence suggests that the source of heterogeneity may be populations of small peptides adherent to the heat shock protein, which may number in the hundreds. *Id.* It has been proposed that a wide diversity of peptides adherent to tumor-synthesized heat shock proteins may render such proteins capable of eliciting an immune response in subjects having diverse HLA phenotypes, in contrast to more traditional immunogens which may be somewhat HLA-restricted in their efficacy. *Id.*

[0007] Recently, Nieland et al. (*Proc. Natl. Acad. Sci. U.S.A.* 93:6135-6139 (1996)) identified an antigenic peptide containing a cytotoxic T lymphocyte (CTL) vesicular stomatitis virus (VSV) epitope bound to gp96 produced by VSV-infected cells. Neiland's methods precluded the identification of any additional peptides or other compounds which

may also have bound to gp96, and were therefore unable to further characterize higher molecular weight material which was bound to gp96 and detected by high pressure liquid chromatography.

[0008] It has been reported that a synthetic peptide comprising multiple iterations of NANP (Asn Ala Asn Pro) (SEQ ID NO:1) malarial antigen, chemically cross-linked to glutaraldehyde-fixed mycobacterial hsp65 or hsp70, was capable of inducing antibody formation (i.e., a humoral response) in mice in the absence of any added adjuvant; a similar effect was observed using heat shock protein from the bacterium *Escherichia coli*. Del Guidice, *Experientia* 50:1061-1066 (1994); Barrios et al., *Clin. Exp. Immunol.* 98:224-228 (1994); Barrios et al., *Eur. J. Immunol.* 22:1365-1372 (1992). Cross-linking of synthetic peptide to heat shock protein and possibly glutaraldehyde fixation was required for antibody induction. Barrios et al., *Clin. Exp. Immunol.* 98:229-233.

[0009] PCT/US96/13363 describes hybrid antigens comprising an antigenic domain and a heat shock protein binding domain that, in a complex with a heat shock protein, induces immunological responses to antigens and are thus useful for treatment of cancer and infectious diseases. PCT/US98/22335 describes additional heat shock protein binding domains for similar uses. It has now been discovered that improvements in the heat shock protein binding domains leads to an increase in biological activity, and thus an increase in inducing an immune response against the antigenic portion of the hybrid antigen, as well as prevention and treatment of diseases associated with the antigenic domains. It is towards these improved heat shock protein binding domains that the present application is directed.

SUMMARY OF THE INVENTION

[0010] The present invention relates to methods and compositions for inducing an immune response in a subject, wherein at least one defined hybrid antigen optionally in a complex with a heat shock protein is administered to the subject. The hybrid antigen comprises an antigenic domain and a heat shock protein binding domain. Induction of an immune response to an antigen associated with a disease such as an infectious disease or tumor is useful for treatment of the disease. The antigenic or immunogenic domain of the hybrid antigen may be an entire protein or peptide antigen, or may be only a portion of the

selected antigen, for example a selected epitope of the antigen. In specific, non-limiting embodiments of the invention, the heat shock protein binding domain comprises a peptide having the sequence:

Asn Leu Leu Arg Leu Thr Gly Trp (SEQ ID NO:417),

5 Phe Tyr Gln Leu Ala Leu Tyr Trp (SEQ ID NO:418), or

Arg Lys Leu Phe Phe Asn Leu Arg Trp (SEQ ID NO:419).

[0011] In alternate embodiments, the heat shock protein binding domain comprises a peptide have a sequence among SEQ ID NOs:132-185 and 193-356.

[0012] The present invention provides for methods of administering such hybrid
10 antigens alone, as well as heat shock protein/hybrid antigen compositions, the latter comprising (i) combining one or more heat shock protein with one or more hybrid antigens *in vitro*, under conditions wherein binding of hybrid antigen to heat shock protein occurs to form a hybrid antigen/heat shock protein complex; and (ii) administering the hybrid antigen, bound to heat shock protein, in an effective amount to a subject in need of such treatment.

15 [0013] Alternatively, hybrid antigens optionally in combination with heat shock protein may be introduced into a subject by administering to the subject a nucleic acid encoding the hybrid antigen, optionally with nucleic acid encoding the heat shock protein.

[0014] Thus, in a first aspect, the invention is directed to a hybrid antigen consisting essentially of an antigenic domain of an infectious agent or tumor antigen, a binding domain
20 that non-covalently binds to a heat shock protein, and a peptide linker separating the antigenic domain and the binding domain, and wherein the binding domain comprises Asn Leu Leu Arg Leu Thr Gly Trp (SEQ ID NO:417), Phe Tyr Gln Leu Ala Leu Tyr Trp (SEQ ID NO:418), Arg Lys Leu Phe Phe Asn Leu Arg Trp (SEQ ID NO:419), or any of SEQ ID NOs:132-185 and 193-356.

25 [0015] In a second aspect, the invention is directed to a hybrid antigen consisting essentially of a plurality of antigenic domains of an infectious agent or tumor antigen, a binding domain that non-covalently binds to a heat shock protein, and peptide linkers separating the antigenic domains and the binding domain, and wherein the binding domain comprises Asn Leu Leu Arg Leu Thr Gly Trp (SEQ ID NO:417), Phe Tyr Gln Leu Ala Leu
30 Tyr Trp (SEQ ID NO:418), Arg Lys Leu Phe Phe Asn Leu Arg Trp (SEQ ID NO:419), or

any of SEQ ID NOs:132-185 and 193-356. In a particular embodiment, at least one of the antigenic domains in the aforementioned hybrid antigen is a T helper epitope.

[0016] In a third aspect, the invention is directed to a hybrid antigen comprising an antigenic domain of an infectious agent or tumor antigen and a binding domain that non-covalently binds to a heat shock protein, and wherein the binding domain comprises Asn Leu Leu Arg Leu Thr Gly Trp (SEQ ID NO:417), Phe Tyr Gln Leu Ala Leu Tyr Trp (SEQ ID NO:418), Arg Lys Leu Phe Phe Asn Leu Arg Trp (SEQ ID NO:419), or any of SEQ ID NOs:132-185 and 193-356. In a particular embodiment, the aforementioned hybrid antigen has a peptide linker separating the antigenic domain and the binding domain.

[0017] In a fourth aspect, the invention is directed to a hybrid antigen comprising a plurality of antigenic domains of an infectious agent or tumor antigen and a binding domain that non-covalently binds to a heat shock protein, and wherein the binding domain comprises Asn Leu Leu Arg Leu Thr Gly Trp (SEQ ID NO:417), Phe Tyr Gln Leu Ala Leu Tyr Trp (SEQ ID NO:418), Arg Lys Leu Phe Phe Asn Leu Arg Trp (SEQ ID NO:419), or any of SEQ ID NOs:132-185 and 193-356. In a particular embodiment, peptide linkers separate the antigenic domains and the binding domain. In yet another embodiment, at least one of the antigenic domains is a T helper epitope.

[0018] In a fifth aspect, the invention is directed to a composition for inducing an immune response to an infectious agent or tumor antigen comprising at least one hybrid antigen, the hybrid antigen comprising an antigenic domain of the infectious agent or tumor antigen and a binding domain that non-covalently binds to a heat shock protein, and wherein the binding domain comprises Asn Leu Leu Arg Leu Thr Gly Trp (SEQ ID NO:417), Phe Tyr Gln Leu Ala Leu Tyr Trp (SEQ ID NO:418), Arg Lys Leu Phe Phe Asn Leu Arg Trp (SEQ ID NO:419), or any of SEQ ID NOs:132-185 and 193-356. In one embodiment, a peptide linker separates the antigenic domain and the binding domain. In another embodiment, the composition comprises a plurality of hybrid antigens, and one of the hybrid antigens can comprise a T helper epitope.

[0019] In a sixth aspect, the invention is directed to a composition for inducing an immune response to an infectious agent or tumor antigen comprising at least one hybrid antigen, the hybrid antigen comprising a plurality of antigenic domains at least one of which

is from the infectious agent or tumor antigen, and a binding domain that non-covalently binds to a heat shock protein, wherein the binding domain comprises Asn Leu Leu Arg Leu Thr Gly Trp (SEQ ID NO:417), Phe Tyr Gln Leu Ala Leu Tyr Trp (SEQ ID NO:418), Arg Lys Leu Phe Phe Asn Leu Arg Trp (SEQ ID NO:419), or any of SEQ ID NOs:132-185 and 193-356. In one embodiment, peptide linkers separate the antigenic domains from the binding domain. In another embodiment, at least one of the antigenic domains comprises a T helper epitope.

[0020] In a seventh aspect, the invention is directed to a composition for inducing an immune response to an infectious agent or tumor antigen comprising at least one hybrid antigen, the hybrid antigen consisting essentially of an antigenic domain of an infectious agent or tumor antigen, a binding domain that non-covalently binds to a heat shock protein, and a peptide linker separating the antigenic domain and the binding domain, and wherein the binding domain comprises Asn Leu Leu Arg Leu Thr Gly Trp (SEQ ID NO:417), Phe Tyr Gln Leu Ala Leu Tyr Trp (SEQ ID NO:418), Arg Lys Leu Phe Phe Asn Leu Arg Trp (SEQ ID NO:419), or any of SEQ ID NOs:132-185 and 193-356. In one embodiment, the aforementioned composition comprises a plurality of hybrid antigens. In another aspect, at least one of the plurality of hybrid antigens comprises a T helper epitope.

[0021] In an eighth aspect, the invention is directed to a composition for inducing an immune response to an infectious agent or tumor antigen comprising at least one hybrid antigen, the hybrid antigen consisting essentially of a plurality of antigenic domains at least one of which is from an infectious agent or tumor antigen, a binding domain that non-covalently binds to a heat shock protein, and a peptide linker separating the antigenic domain and the binding domain, and wherein the binding domain comprises Asn Leu Leu Arg Leu Thr Gly Trp (SEQ ID NO:417), Phe Tyr Gln Leu Ala Leu Tyr Trp (SEQ ID NO:418), Arg Lys Leu Phe Phe Asn Leu Arg Trp (SEQ ID NO:419), or any of SEQ ID NOs:132-185 and 193-356. In one embodiment, at least one of the antigenic domains comprises a T helper epitope.

[0022] In a ninth aspect, the invention is directed to a method for inducing an immune response to an infectious agent or tumor antigen comprising administering to a subject a complex of:

(a) a hybrid antigen comprising at least one antigenic domain of the infectious agent or tumor antigen, and a binding domain comprising a peptide that non-covalently binds to a heat shock protein; and

(b) a heat shock protein;

5 wherein the hybrid antigen and the heat shock protein are non-covalently bound, and wherein the binding domain comprises Asn Leu Leu Arg Leu Thr Gly Trp (SEQ ID NO:417), Phe Tyr Gln Leu Ala Leu Tyr Trp (SEQ ID NO:418), Arg Lys Leu Phe Phe Asn Leu Arg Trp (SEQ ID NO:419), or any of SEQ ID NOs:132-185 and 193-356. In one embodiment, the complex comprises a plurality of hybrid antigens. In an embodiment, at
10 least one of the hybrid antigens is a T helper epitope. In another embodiment, the hybrid antigen comprises a plurality of antigenic domains, and at least one of the antigenic domains can be a T helper epitope. In yet another embodiment wherein the complex comprises a plurality of hybrid antigens, at least one of the hybrid antigens comprises a plurality of antigenic domains. In an embodiment of this aspect of the invention, a peptide
15 linker separates the antigenic domain and the binding domain. In another embodiment of this aspect of the invention, the heat shock protein is a hsp70.

[0023] In a tenth aspect, the invention is directed to a method for inducing an immune response to an infectious agent or tumor antigen comprising administering to a subject a complex of a heat shock protein and a hybrid antigen, the hybrid antigen consisting
20 essentially of at least one antigenic domain of an infectious agent or tumor antigen, a binding domain that non-covalently binds to a heat shock protein, and a peptide linker separating the antigenic domain and the binding domain, and wherein the binding domain comprises Asn Leu Leu Arg Leu Thr Gly Trp (SEQ ID NO:417), Phe Tyr Gln Leu Ala Leu Tyr Trp (SEQ ID NO:418), Arg Lys Leu Phe Phe Asn Leu Arg Trp (SEQ ID NO:419), or
25 any of SEQ ID NOs:132-185 and 193-356. In one embodiment, the complex comprises a plurality of hybrid antigens. In another embodiment, at least one of the hybrid antigens is a T helper epitope. In a further embodiment, the hybrid antigen comprises a plurality of antigenic domains. In yet a further embodiment, at least one of the antigenic domains is a T helper epitope. In still yet another embodiment, the complex comprises a plurality of hybrid
30 antigens, at least one of the hybrid antigens comprising a plurality of antigenic domains. In a preferred embodiment of this aspect, the heat shock protein is a hsp70.

[0024] In an eleventh aspect, the invention is directed to a method for inducing an immune response to an infectious agent or tumor antigen comprising administering to a subject at least one hybrid antigen comprising at least one antigenic domain of the infectious agent or tumor antigen, and a binding domain comprising a peptide that non-covalently binds to a heat shock protein, wherein the binding domain comprises Asn Leu Leu Arg Leu Thr Gly Trp (SEQ ID NO:417), Phe Tyr Gln Leu Ala Leu Tyr Trp (SEQ ID NO:418), Arg Lys Leu Phe Phe Asn Leu Arg Trp (SEQ ID NO:419), or any of SEQ ID NOs:132-185 and 193-356. In one embodiment, the complex comprises a plurality of hybrid antigens. In another embodiment, at least one of the hybrid antigens is a T helper epitope. In another embodiment, the hybrid antigen comprises a plurality of antigenic domains. In a further embodiment, at least one of the antigenic domains is a T helper epitope. In yet a further embodiment, the complex comprises a plurality of hybrid antigens, at least one of the hybrid antigens comprising a plurality of antigenic domains. In another embodiment of this aspect of the invention, a peptide linker separates the antigenic domain and the binding domain.

[0025] In a twelfth embodiment, the invention is directed to a method for inducing an immune response to an infectious agent or tumor antigen comprising administering to a subject at least one hybrid antigen, the hybrid antigen consisting essentially of at least one antigenic domain of an infectious agent or tumor antigen, a binding domain that non-covalently binds to a heat shock protein, and a peptide linker separating the antigenic domain and the binding domain, and wherein the binding domain comprises Asn Leu Leu Arg Leu Thr Gly Trp (SEQ ID NO:417), Phe Tyr Gln Leu Ala Leu Tyr Trp (SEQ ID NO:418), Arg Lys Leu Phe Phe Asn Leu Arg Trp (SEQ ID NO:419), or any of SEQ ID NOs:132-185 and 193-356. In one embodiment, the complex comprises a plurality of hybrid antigens. In a further embodiment, at least one of the hybrid antigens is a T helper epitope. In another embodiment, the hybrid antigen comprises a plurality of antigenic domains. In yet another embodiment, at least one of the antigenic domains is a T helper epitope. In yet still a further embodiment, the complex comprises a plurality of hybrid antigens, at least one of the hybrid antigens comprising a plurality of antigenic domains.

[0026] In a thirteenth aspect, the invention is directed to a method for treating an infectious disease or cancer comprising administering to a subject a complex of:

(a) a hybrid antigen comprising at least one antigenic domain of an infectious agent or tumor antigen associated with the infectious disease or cancer, and a binding domain comprising a peptide that non-covalently binds to a heat shock protein; and

5 (b) a heat shock protein;

wherein the hybrid antigen and the heat shock protein are non-covalently bound, and wherein the binding domain comprises Asn Leu Leu Arg Leu Thr Gly Trp (SEQ ID NO:417), Phe Tyr Gln Leu Ala Leu Tyr Trp (SEQ ID NO:418), Arg Lys Leu Phe Phe Asn Leu Arg Trp (SEQ ID NO:419), or any of SEQ ID NOs:132-185 and 193-356. In one
10 embodiment, the complex comprises a plurality of hybrid antigens. In another embodiment, at least one of the hybrid antigens is a T helper epitope. In yet another embodiment, the hybrid antigen comprises a plurality of antigenic domains. In still another embodiment, at least one of the antigenic domains is a T helper epitope. In yet still a further embodiment, the complex comprises a plurality of hybrid antigens, at least one of the hybrid antigens
15 comprising a plurality of antigenic domains. In an embodiment of this aspect of the invention, a peptide linker separates the antigenic domain and the binding domain. In a preferred embodiment of this aspect of the invention, the heat shock protein is a hsp70.

[0027] In a fourteenth aspect, the invention is directed to a method for treating an infectious disease or cancer comprising administering to a subject a complex of a heat shock
20 protein and a hybrid antigen, the hybrid antigen consisting essentially of at least one antigenic domain of an infectious agent or tumor antigen associated with the infectious disease or cancer, a binding domain that non-covalently binds to a heat shock protein, and a peptide linker separating the antigenic domain and the binding domain, and wherein the binding domain comprises Asn Leu Leu Arg Leu Thr Gly Trp (SEQ ID NO:417), Phe Tyr
25 Gln Leu Ala Leu Tyr Trp (SEQ ID NO:418), Arg Lys Leu Phe Phe Asn Leu Arg Trp (SEQ ID NO:419), or any of SEQ ID NOs:132-185 and 193-356. In one embodiment, the complex comprises a plurality of hybrid antigens. In another aspect, at least one of the hybrid antigens is a T helper epitope. In yet another aspect, the hybrid antigen comprises a plurality of antigenic domains. In yet another aspect, at least one of the antigenic domains
30 is a T helper epitope. In a further aspect, the complex comprises a plurality of hybrid

antigens, at least one of the hybrid antigens comprising a plurality of antigenic domains. In a preferred embodiment, the heat shock protein is a hsp70.

[0028] In a fifteen aspect, the invention is directed to a method for treating an infectious disease or cancer comprising administering to a subject at least one hybrid antigen
5 comprising at least one antigenic domain of an infectious agent or tumor antigen associated with the infectious disease or cancer, and a binding domain comprising a peptide that non-covalently binds to a heat shock protein, wherein the binding domain comprises Asn Leu Leu Arg Leu Thr Gly Trp (SEQ ID NO:417), Phe Tyr Gln Leu Ala Leu Tyr Trp (SEQ ID NO:418), Arg Lys Leu Phe Phe Asn Leu Arg Trp (SEQ ID NO:419), or any of SEQ ID
10 NOs:132-185 and 193-356. In one embodiment, the complex comprises a plurality of hybrid antigens. In another aspect, at least one of the hybrid antigens is a T helper epitope. In yet another aspect, the hybrid antigen comprises a plurality of antigenic domains. In still a further aspect, at least one of the antigenic domains is a T helper epitope. In still yet another aspect, the complex comprises a plurality of hybrid antigens, at least one of the
15 hybrid antigens comprising a plurality of antigenic domains. In one embodiment of this aspect of the invention, a peptide linker separates the antigenic domain and the binding domain.

[0029] In a sixteenth aspect, the invention is directed to a method for treating an infectious disease or cancer comprising administering to a subject at least one hybrid
20 antigen, the hybrid antigen consisting essentially of at least one antigenic domain of an infectious agent or tumor antigen associated with an infectious disease or cancer, a binding domain that non-covalently binds to a heat shock protein, and a peptide linker separating the antigenic domain and the binding domain, and wherein the binding domain comprises Asn Leu Leu Arg Leu Thr Gly Trp (SEQ ID NO:417), Phe Tyr Gln Leu Ala Leu Tyr Trp (SEQ
25 ID NO:418), Arg Lys Leu Phe Phe Asn Leu Arg Trp (SEQ ID NO:419), or any of SEQ ID NOs:132-185 and 193-356. In one embodiment, the complex comprises a plurality of hybrid antigens. In another embodiment, at least one of the hybrid antigens is a T helper epitope. In yet another embodiment, the hybrid antigen comprises a plurality of antigenic domains. In still yet another embodiment, at least one of the antigenic domains is a T
30 helper epitope. In another embodiment, the complex comprises a plurality of hybrid antigens, at least one of the hybrid antigens comprising a plurality of antigenic domains.

[0030] In a seventeenth aspect, the invention is directed to a peptide comprising Asn Leu Leu Arg Leu Thr Gly Trp (SEQ ID NO:417), Phe Tyr Gln Leu Ala Leu Tyr Trp (SEQ ID NO:418), Arg Lys Leu Phe Phe Asn Leu Arg Trp (SEQ ID NO:419), or any of SEQ ID NOs:132-185 and 193-356.

5 [0031] In an eighteenth aspect, the invention is directed to an immunogenic polypeptide comprising a plurality of antigenic domains, and a binding domain comprises Asn Leu Leu Arg Leu Thr Gly Trp (SEQ ID NO:417), Phe Tyr Gln Leu Ala Leu Tyr Trp (SEQ ID NO:418), Arg Lys Leu Phe Phe Asn Leu Arg Trp (SEQ ID NO:419), or any of SEQ ID NOs:132-185 and 193-356.

10 [0032] In a nineteenth aspect, the invention is directed to the peptides Asn Leu Leu Arg Leu Thr Gly Trp (SEQ ID NO:417), Phe Tyr Gln Leu Ala Leu Tyr Trp (SEQ ID NO:418), Arg Lys Leu Phe Phe Asn Leu Arg Trp (SEQ ID NO:419), or any of SEQ ID NOs:132-185 and 193-356.

15 [0033] In a twentieth aspect, the invention is directed to a polynucleotide encoding any of the aforementioned hybrid antigens.

[0034] In a twenty-first aspect, the invention is directed to a method of inducing an immune response to an infectious disease or cancer comprising administering to a subject a polynucleotide encoding a hybrid antigen comprising an antigenic domain of an infectious agent or tumor antigen and a heat shock protein binding domain.

20 [0035] In a twenty-second aspect, the invention is directed a method of inducing an immune response to an infectious disease or cancer comprising administering to a subject a polynucleotide encoding a hybrid antigen as entioned above, and a polynucleotide encoding a heat shock protein. In a preferred embodiment, the encoded heat shock protein is a hsp70.

25 [0036] In a twenty-third aspect, the invention is directed to polynucleotides encoding Asn Leu Leu Arg Leu Thr Gly Trp (SEQ ID NO:417), Phe Tyr Gln Leu Ala Leu Tyr Trp (SEQ ID NO:418), Arg Lys Leu Phe Phe Asn Leu Arg Trp (SEQ ID NO:419), or any of SEQ ID NOs:132-185 and 193-356. In a further embodiment, the invention is directed to polynucleotides encoding hybrid antigens as described above. In another embodiment, the invention is directed to inducing an immune response to an infectious agent or cancer

comprising administering to a subject a polynucleotide encoding a hybrid antigen as mentioned above, optionally together with a polynucleotide encoding a heat shock protein, preferably hsp70. In a further embodiment, the invention is directed to treating an infectious disease or cancer comprising administering to a subject a polynucleotide
5 encoding a hybrid antigen as mentioned above, optionally together with a polynucleotide encoding a heat shock protein, preferably hsp70.

[0037] In any or all of the aforementioned aspects of the invention, the infectious disease antigen may be derived from an infectious agent such as a bacterium, virus, protozoan, mycoplasma, fungus, yeast, parasite, or prion, by way of non-limiting example.

10 A cancer or tumor antigen associated with cancer may be derived from a sarcoma, a lymphoma, a leukemia, or a carcinoma, a melanoma, carcinoma of the breast, carcinoma of the prostate, ovarian carcinoma, carcinoma of the cervix, colon carcinoma, carcinoma of the lung, glioblastoma, or astrocytoma, by way of non-limiting examples. The antigenic domain of an infectious agent or cancer comprises an antigen derived from or associated
15 with the infectious disease or tumor antigen, such that induction of an immune response to the antigen of the infectious agent or cancer antigen, respectively, is useful for treating the corresponding infectious disease or cancer.

[0038] This application claims priority under 35 U.S.C. § 119(e) to provisional applications 60/447,142, filed February 13, 2003; 60/462,469, filed April 11, 2003;
20 60/463,746, filed April 18, 2003; and 60/503,417, filed September 16, 2003, all four of which are incorporated herein by reference in their entireties.

BRIEF DESCRIPTION OF THE DRAWINGS

[0039] Figure 1 shows the induction of an in-vivo immune response with several hybrid
25 antigens of the invention in a complex with hsp70.

[0040] Figure 2 shows the induction of an in-vitro immune response using various hybrid antigens of the invention in a complex with hsp70.

[0041] Figure 3 shows Hill plots for determining the affinity of various peptides for hsp70.

[0042] Figures 4a and 4b show the results of an in-vitro macrophage T-cell activation assay using various hybrid antigens of the invention alone or in a complex with hsp70.

5 [0043] Figure 5 shows in-vivo responses to complexes of hybrid antigens of the invention alone or in a complex with hsp70.

[0044] Figure 6 shows the blocking of in-vivo responses to one hybrid antigen-hsp70 complex with the addition of a heat shock protein binding domain peptide alone.

10 [0045] Figure 7 shows that smaller doses of a higher affinity heat shock protein binding domain-epitope in a complex with hsp70 can elicit immune responses in vivo.

DETAILED DESCRIPTION OF THE INVENTION

[0046] For purposes of clarity of description, and not by way of limitation, the detailed description is divided into the following subsections:

- 15 (i) hybrid antigens,
(ii) heat shock proteins; and
(iii) methods of administration.

Hybrid Antigens

20 [0047] A hybrid antigen, according to the invention comprises an immunogenic (antigenic) domain as well as a heat shock protein-binding domain. An optional linker, preferably a peptide linker, may be provided between these domains. Thus, the hybrid antigen serves at least two functions, namely (i) it contains an epitope capable of inducing the desired immune response; and (ii) it is capable of physically binding to a heat shock protein. As will be noted below, such binding may occur in vivo such that administration of
25 the hybrid antigen alone will induce the desired immune response and provide the desired therapeutic effect.

[0048] The term "antigen" as used herein, refers to a compound which may be composed of amino acids, carbohydrates, nucleic acids or lipids individually or in any combination.

[0049] The term "hybrid antigen," as used herein, refers to a compound which binds to one or more heat shock proteins and which is representative of the immunogen toward which an immune response is desirably directed. For example, where the immunogen is an influenza virus, the hybrid antigen may comprise a peptide fragment of the matrix protein of the influenza virus. As used herein, the term "immunogen" is applied to the neoplastic cell, infected cell, pathogen, or component thereof, towards which an immune response is to be elicited, whereas the hybrid antigen comprises a portion of that immunogen which can provoke the desired response and which binds to one or more heat shock proteins. In particular, the antigenic domain of the hybrid antigen is selected to elicit an immune response to a particular disease or pathogen, including peptides obtained from MHC molecules, mutated DNA gene products, and direct DNA products such as those obtained from tumor cells.

[0050] While the invention may be applied to any type of immunogen, immunogens of particular interest are those associated with, derived from, or predicted to be associated with a neoplastic disease, including but not limited to a sarcoma, a lymphoma, a leukemia, or a carcinoma, and in particular, with melanoma, carcinoma of the breast, carcinoma of the prostate, ovarian carcinoma, carcinoma of the cervix, colon carcinoma, carcinoma of the lung, glioblastoma, astrocytoma, etc. Selections of melanoma antigens useful in hybrid antigens of the present invention may be found, by way of non-limiting example, in PCT/US01/12449 (WO0178655), incorporated herein by reference in its entirety. Further, mutations of tumor suppressor gene products such as p53, or oncogene products such as ras may also provide hybrid antigens to be used according to the invention.

[0051] In further embodiments, the immunogen may be associated with an infectious disease, and, as such, may be a bacterium, virus, protozoan, mycoplasma, fungus, yeast, parasite, or prion. For example, but not by way of limitation, the immunogen may be a human papilloma virus (see below), a herpes virus such as herpes simplex or herpes zoster, a retrovirus such as human immunodeficiency virus 1 or 2, a hepatitis virus, an influenza virus, a rhinovirus, respiratory syncytial virus, cytomegalovirus, adenovirus, *Mycoplasma*

pneumoniae, a bacterium of the genus *Salmonella*, *Staphylococcus*, *Streptococcus*, *Enterococcus*, *Clostridium*, *Escherichia*, *Klebsiella*, *Vibrio*, *Mycobacterium*, amoeba, a malarial parasite, *Trypanosoma cruzi*, etc.

[0052] Immunogens may be obtained by isolation directly from a neoplasm, an infected cell, a specimen from an infected subject, a cell culture, or an organism culture, or may be synthesized by chemical or recombinant techniques. Suitable antigenic peptides, particularly for use in a hybrid antigen, for use against viruses, bacteria and the like can be designed by searching through their sequences for MHC class I restricted peptide epitopes containing HLA binding sequences such as but not limited to HLA-A2 peptide binding sequences:

Xaa(Leu/Met)XaaXaaXaa(Val/Ile/Leu/Thr)XaaXaa(Val/Leu) (SEQ ID NO:2), for example,

from viruses:

Ser Gly Pro Ser Asn Thr Pro Pro Glu Ile (SEQ ID NO:10);

Ser Gly Val Glu Asn Pro Gly Gly Tyr Cys Leu (SEQ ID NO:11);

Lys Ala Val Tyr Asn Phe Ala Thr Cys Gly (SEQ ID NO:12);

Arg Pro Gln Ala Ser Gly Val Tyr Met (SEQ ID NO:13);

Phe Gln Pro Gln Asn Gly Gln Phe Ile (SEQ ID NO:14);

Ile Glu Gly Gly Trp Thr Gly Met Ile (SEQ ID NO:15);

Thr Tyr Val Ser Val Ser Thr Ser Thr Leu (SEQ ID NO:16);

Phe Glu Ala Asn Gly Asn Leu Ile (SEQ ID NO:17);

Ile Tyr Ser Thr Val Ala Ser Ser Leu (SEQ ID NO:18);

Thr Tyr Gln Arg Thr Arg Ala Leu Val (SEQ ID NO:19);

Cys Thr Glu Leu Lys Leu Ser Asp Tyr (SEQ ID NO:20);

Ser Asp Tyr Glu Gly Arg Leu Ile (SEQ ID NO:21);

Glu Glu Gly Ala Ile Val Gly Glu Ile (SEQ ID NO:22);

Val Ser Asp Gly Gly Pro Asn Leu Tyr (SEQ ID NO:23);

Ala Ser Asn Glu Asn Met Glu Thr Met (SEQ ID NO:24);

Ala Ser Asn Glu Asn Met Asp Ala Met (SEQ ID NO:25);

Lys Leu Gly Glu Phe Tyr Asn Gln Met Met (SEQ ID NO:26);

Leu Tyr Gln Asn Val Gly Thr Tyr Val (SEQ ID NO:27);

Thr Tyr Val Ser Val Gly Thr Ser Thr Leu (SEQ ID NO:28);

Phe Glu Ser Thr Gly Asn Leu Ile (SEQ ID NO:29);
 Val Tyr Gln Ile Leu Ala Ile Tyr Ala (SEQ ID NO:30);
 Ile Tyr Ala Thr Val Ala Gly Ser Leu (SEQ ID NO:31);
 Gly Ile Leu Gly Phe Val Phe Thr Leu (SEQ ID NO:32);
 5 Ile Leu Gly Phe Val Phe Thr Leu Thr Val (SEQ ID NO:33);
 Ile Leu Arg Gly Ser Val Ala His Lys (SEQ ID NO:34);
 Glu Asp Leu Arg Val Leu Ser Phe Ile (SEQ ID NO:35);
 Glu Leu Arg Ser Arg Tyr Trp Ala Ile (SEQ ID NO:36);
 Ser Arg Tyr Trp Ala Ile Arg Thr Arg (SEQ ID NO:37);
 10 Lys Thr Gly Gly Pro Ile Tyr Lys Arg (SEQ ID NO:38);
 Phe Ala Pro Gly Asn Tyr Pro Ala Leu (SEQ ID NO:39);
 Arg Arg Tyr Pro Asp Ala Val Tyr Leu (SEQ ID NO:40);
 Asp Pro Val Ile Asp Arg Leu Tyr Leu (SEQ ID NO:41);
 Ser Pro Gly Arg Ser Phe Ser Tyr Phe (SEQ ID NO:42);
 15 Tyr Pro Ala Leu Gly Leu His Glu Phe (SEQ ID NO:43);
 Thr Tyr Lys Asp Thr Val Gln Leu (SEQ ID NO:44);
 Phe Tyr Asp Gly Phe Ser Lys Val Pro Leu (SEQ ID NO:45);
 Phe Ile Ala Gly Asn Ser Ala Tyr Glu Tyr Val (SEQ ID NO:46);
 Tyr Pro His Phe Met Pro Thr Asn Leu (SEQ ID NO:47);
 20 Ala Pro Thr Ala Gly Ala Phe Phe Phe (SEQ ID NO:48);
 Ser Thr Leu Pro Glu Thr Thr Val Val Arg Arg (SEQ ID NO:49);
 Phe Leu Pro Ser Asp Phe Phe Pro Ser Val (SEQ ID NO:50);
 Trp Leu Ser Leu Leu Val Pro Phe Val (SEQ ID NO:51);
 Gly Leu Ser Pro Thr Val Trp Leu Ser Val (SEQ ID NO:52);
 25 Asp Leu Met Gly Tyr Ile Pro Leu Val (SEQ ID NO:53);
 Leu Met Gly Tyr Ile Pro Leu Val Gly Ala (SEQ ID NO:54);
 Ala Ser Arg Cys Trp Val Ala Met (SEQ ID NO:55);
 Lys Leu Val Ala Leu Gly Ile Asn Ala Val (SEQ ID NO:56);
 Phe Leu Arg Gly Arg Ala Tyr Gly Leu (SEQ ID NO:57);
 30 Arg Arg Ile Tyr Asp Leu Ile Glu Leu (SEQ ID NO:58);
 Ile Val Thr Asp Phe Ser Val Ile Lys (SEQ ID NO:59);
 Arg Arg Arg Trp Arg Arg Leu Thr Val (SEQ ID NO:60);
 Glu Glu Asn Leu Leu Asp Phe Val Arg Phe (SEQ ID NO:61);

Cys Leu Gly Gly Leu Leu Thr Met Val (SEQ ID NO:62);
 Ser Ser Ile Glu Phe Ala Arg Leu (SEQ ID NO:63);
 Leu Tyr Arg Thr Phe Ala Gly Asn Pro Arg Ala (SEQ ID NO:64);
 Asp Tyr Ala Thr Leu Gly Val Gly Val (SEQ ID NO:65);
 5 Leu Leu Leu Gly Thr Leu Asn Ile Val (SEQ ID NO:66);
 Leu Leu Met Gly Thr Leu Gly Ile Val (SEQ ID NO:67);
 Thr Leu Gln Asp Ile Val Leu His Leu (SEQ ID NO:68);
 Gly Leu His Cys Tyr Glu Gln Leu Val (SEQ ID NO:69);
 Pro Leu Lys Gln His Phe Gln Ile Val (SEQ ID NO:70);
 10 Arg Leu Val Thr Leu Lys Asp Ile Val (SEQ ID NO:71);
 Arg Ala His Tyr Asn Ile Val Thr Phe (SEQ ID NO:72);
 Leu Leu Phe Gly Tyr Pro Val Tyr Val (SEQ ID NO:73);
 Ser Ala Ile Asn Asn Tyr Ala Gln Lys Leu (SEQ ID NO:74);
 His Gln Ala Ile Ser Pro Arg Thr Leu (SEQ ID NO:75);
 15 Gln Met Val His Gln Ala Ile Ser Pro Arg Thr Leu (SEQ ID NO:76);
 Cys Lys Gly Val Asn Lys Glu Tyr Leu (SEQ ID NO:77);
 Gln Gly Ile Asn Asn Leu Asp Asn Leu (SEQ ID NO:78);
 Asn Asn Leu Asp Asn Leu Arg Asp Tyr (SEQ ID NO:79);
 Ser Glu Phe Leu Leu Glu Lys Arg Ile (SEQ ID NO:80);
 20 Ser Tyr Ile Gly Ser Ile Asn Asn Ile (SEQ ID NO:81);
 Ile Leu Gly Asn Lys Ile Val Arg Met Tyr (SEQ ID NO:82);
 Arg Leu Arg Pro Gly Gly Lys Lys Lys (SEQ ID NO:83);
 Glu Ile Lys Asp Thr Lys Glu Ala Leu (SEQ ID NO:84);
 Gly Glu Ile Tyr Lys Arg Trp Ile Ile (SEQ ID NO:85);
 25 Glu Ile Tyr Lys Arg Trp Ile Ile Leu (SEQ ID NO:86);
 Arg Tyr Leu Lys Asp Gln Gln Leu Leu (SEQ ID NO:87);
 Arg Gly Pro Gly Arg Ala Phe Val Thr Ile (SEQ ID NO:88);
 Ile Val Gly Leu Asn Lys Ile Val Arg (SEQ ID NO:89);
 Thr Val Tyr Tyr Gly Val Pro Val Trp Lys (SEQ ID NO:90);
 30 Arg Leu Arg Asp Leu Leu Leu Ile Val Thr Arg (SEQ ID NO:91);
 Lys Arg Trp Ile Ile Leu Gly Leu Asn Lys (SEQ ID NO:92);
 Ser Phe Asn Cys Gly Gly Glu Phe Phe (SEQ ID NO:93);
 Gly Arg Ala Phe Val Thr Ile Gly Lys (SEQ ID NO:94);

Thr Pro Gly Pro Gly Val Arg Tyr Pro Leu (SEQ ID NO:95);
 Gln Val Pro Leu Arg Pro Met Thr Tyr Lys (SEQ ID NO:96);
 Thr Glu Met Glu Lys Glu Gly Lys Ile (SEQ ID NO:97);
 Ile Leu Lys Glu Pro Val His Gly Val (SEQ ID NO:98);
 5 Val Glu Ala Glu Ile Ala His Gln Ile (SEQ ID NO:99);
 Arg Gly Tyr Val Tyr Gln Gly Leu (SEQ ID NO:100);
 Tyr Ser Gly Tyr Ile Phe Arg Asp Leu (SEQ ID NO:101);
 Val Gly Pro Val Phe Pro Pro Gly Met (SEQ ID NO:102);
 Ile Ile Tyr Arg Phe Leu Leu Ile (SEQ ID NO:103);
 10 from bacteria:
 Lys Tyr Gly Val Ser Val Gln Asp Ile (SEQ ID NO:104);
 Ile Gln Val Gly Asn Thr Arg Thr Ile (SEQ ID NO:105);
 Thr Pro His Pro Ala Arg Ile Gly Leu (SEQ ID NO:106);
 from parasites:
 15 Ser Tyr Ile Pro Ser Ala Glu Lys Ile (SEQ ID NO:107);
 Lys Pro Lys Asp Glu Leu Asp Tyr (SEQ ID NO:108);
 Lys Ser Lys Asp Glu Leu Asp Tyr (SEQ ID NO:109);
 Lys Pro Asn Asp Lys Ser Leu Tyr (SEQ ID NO:110);
 Lys Tyr Leu Lys Lys Ile Lys Asn Ser Leu (SEQ ID NO:111);
 20 Tyr Glu Asn Asp Ile Glu Lys Lys Ile (SEQ ID NO:112);
 Asn Tyr Asp Asn Ala Gly Thr Asn Leu (SEQ ID NO:113);
 Asp Glu Leu Asp Tyr Glu Asn Asp Ile (SEQ ID NO:114);
 Ser Tyr Val Pro Ser Ala Glu Gln Ile (SEQ ID NO:115);
 from cancers:
 25 Phe Glu Gln Asn Thr Ala Gln Pro (SEQ ID NO:116);
 Phe Glu Gln Asn Thr Ala Gln Ala (SEQ ID NO:117);
 Glu Ala Asp Pro Thr Gly His Ser Tyr (SEQ ID NO:118);
 Glu Val Asp Pro Ile Gly His Leu Tyr (SEQ ID NO:119);
 Ala Ala Gly Ile Gly Ile Leu Thr Val (SEQ ID NO:120);
 30 Tyr Leu Glu Pro Gly Pro Val Thr Ala (SEQ ID NO:121);
 Ile Leu Asp Gly Thr Ala Thr Leu Arg Leu (SEQ ID NO:122);
 Met Leu Leu Ala Leu Leu Tyr Cys Leu (SEQ ID NO:123);
 Tyr Met Asn Gly Thr Met Ser Gln Val (SEQ ID NO:124);

Leu Pro Tyr Leu Gly Trp Leu Val Phe (SEQ ID NO:125);
Phe Gly Pro Tyr Lys Leu Asn Arg Leu (SEQ ID NO:126);
Lys Ser Pro Trp Phe Thr Thr Leu (SEQ ID NO:127);
Gly Pro Pro His Ser Asn Asn Phe Gly Tyr (SEQ ID NO:128); and
5 Ile Ser Thr Gln Asn His Arg Ala Leu (SEQ ID NO:129)
(Rammensee et al., *Immunogenetics* 41:178-223 (1995)),
Xaa(Leu/Met)XaaXaaXaaXaaXaaXaaVal (SEQ ID NO:3)
(Tarpey et al., *Immunology* 81:222-227 (1994)),
Xaa(Val/Gln)XaaXaaXaaXaaXaaXaaLeu (SEQ ID NO:4),

10 for example, from virus:

Tyr Gly Ile Leu Gly Lys Val Phe Thr Leu (SEQ ID NO:130);
Ser Leu Tyr Asn Thr Val Ala Thr Leu (SEQ ID NO:131);
(Barouch et al., *J. Exp. Med.* 182:1847-1856 (1995)).

[0053] The foregoing epitopes are merely exemplary of selections available associated
15 with various infectious diseases and cancer, and are provided without any intent whatsoever
to be limiting.

[0054] It may also be desirable to consider the type of immune response which is
desired. For example, under certain circumstances, a humoral immune response may be
appropriate. In other cases, and indeed where an immune response directed toward
20 neoplastic cells or infected cells is sought to be elicited, a cellular immune response is
particularly desirable. Accordingly, particular epitopes associated with the activation of B
cells, T helper cells, or cytotoxic T cells may be identified and selected for incorporation
into the hybrid antigen.

[0055] It may also be desirable to utilize hybrid antigen associated with an autoimmune
25 disease or allergy. Such a hybrid antigen may be administered, together with one or more
heat shock proteins, in an amount sufficient to be tolerogenic or to inhibit a pre-existing
immune response to the hybrid antigen in a subject. The amount of heat shock protein
required to inhibit the immune response is expected to be substantially greater than the
amount required for stimulation.

[0056] Although the size of hybrid antigen may vary depending upon the heat shock protein used, in non-limiting embodiments of the invention, the hybrid antigen may be the size of a peptide having between 10 and 500 amino acid residues, and preferably be the size of a peptide having between 14 and 100, most preferably 18 and 50 amino acid residues.

5 As such, it may be desirable to produce a fragment of an immunogen to serve as a hybrid antigen, or, alternatively, to synthesize a hybrid antigen by chemical or recombinant DNA methods.

[0057] Based on the foregoing considerations, a hybrid antigen may be prepared, and then tested for its ability to bind to heat shock protein. In some instances, binding of hybrid
10 antigen to a particular heat shock protein may be facilitated by the presence of at least one other protein, which may be a heat shock protein.

[0058] For example, binding of hybrid antigen to a heat shock protein may be evaluated by labeling the hybrid antigen with a detectable label, such as a radioactive, fluorescent, enzymatic or pigmented label, combining the hybrid antigen with heat shock protein under
15 conditions which would be expected to permit binding to occur, and then isolating the heat shock protein while removing any unbound hybrid antigen, and determining whether any labeled hybrid antigen had adhered to the heat shock protein. As a specific example, and not by way of limitation, the ability of a hybrid antigen to bind to BiP heat shock protein may be evaluated by combining 2 μ g BiP with up to about 1150 pmole of radioactively
20 labeled hybrid antigen in buffer containing 50 mM Tris HCl (pH 7.5), 200 mM NaCl, and 1 mM Na₂EDTA, in a final volume of 50 μ l, for 30 minutes at 37 degrees Centigrade. Unbound hybrid antigen may then be removed from bound BiP-hybrid antigen by centrifugation at 100g by desalting through a 1 ml Sephadex-G column for 2 minutes. Penefsky, *J. Biol. Chem.* 252:2891 (1977). To prevent binding to the resin, columns may
25 first be treated with 100 μ l of bovine serum albumin in the same buffer and centrifuged as above. Bound hybrid antigen may then be quantitated by liquid scintillation counting. See Flynn et al., *Science* 245:385-390 (1989).

[0059] Because ATP hydrolysis drives the release of peptides from many known heat shock proteins, the amount of ATPase activity may often be used to quantitate the amount
30 of hybrid antigen binding to heat shock protein. An example of how such an assay may be performed is set forth in Flynn et al., *Science* 245:385-390 (1989).

[0060] The heat shock protein-binding domain is selected so that the hybrid antigen will bind *in vitro* or *in vivo* to a heat shock protein such as BiP, hsp70, gp96, or hsp90, or a member of the foregoing heat shock protein families, alone or in combination with accessory heat shock proteins such as hsp40, or hsp60. Peptides which fulfill this criterion
5 may be identified initially by panning libraries of antigens known to bind well to one or more heat shock proteins as described in Blond-Elguindi et al., *Cell* 75:717-728 (1993). Using this technique, Blond-Elguindi have concluded that the heat shock protein BiP recognizes polypeptides that contain a heptameric region having the sequence

Hy(Trp/X)HyXHyXHy (SEQ ID NO:7)

10 where Hy represents a hydrophobic amino acid residue, particularly tryptophan, leucine or phenylalanine (SEQ ID NO:8), and X is any amino acid.

[0061] Other heat shock protein binding motifs have also been identified. For example, Auger et al., *Nature Medicine* 2:306-310 (1996) have identified two pentapeptide binding motifs

15 Gln Lys Arg Ala Ala (SEQ ID NO:5) and

Arg Arg Arg Ala Ala (SEQ ID NO:6)

in HLA-DR types associated with rheumatoid arthritis which bind to heat shock proteins.

Heat shock binding motifs have also been identified as consisting of seven to fifteen residue long peptides which are enriched in hydrophobic amino acids.

20 (Gragerov et al., *J. Molec. Biol.* 235:848-854 (1994)).

[0062] It has been found that incorporation of a tryptophan residue (Trp, or single amino acid code W) at the C-terminus of the heat shock protein binding domains such as but not limited to those identified as described above, enhances binding to heat shock proteins. Increased binding to heat shock proteins has been found to increase the ability of
25 hybrid antigens to induce an immune response to the antigenic domain of the hybrid antigen, whether administered in a complex with a heat shock protein or when administered alone. Increased immune response is correlated with increased efficacy of treating disease.

[0063] Moreover, the addition of a tryptophan residue to the heat shock protein binding domain renders unto such peptides the ability to be detected or be better detected using
30 ultraviolet light absorbance, and the detectability permits facile evaluation of the binding of the peptides to heat shock proteins, by methods such as but not limited to those described

herein. Other examples of methods for determining affinity are described in PCT/US96/13363 (WO9706821), which is incorporated herein by reference in its entirety.

[0064] Non-limiting examples of such heat shock protein binding domains with a terminal Trp residue useful for the various aspects of the present invention include:

- 5 [0065] Asn Leu Leu Arg Leu Thr Gly Trp (SEQ ID NO:417);
Phe Tyr Gln Leu Ala Leu Tyr Trp (SEQ ID NO:418);
Arg Lys Leu Phe Phe Asn Leu Arg Trp (SEQ ID NO:419);
Gly Lys Trp Val Tyr Ile Gly Trp (SEQ ID NO:132);
Ala Lys Arg Glu Thr Lys Gly Trp (SEQ ID NO:133);
10 Lys Trp Val His Leu Phe Gly Trp (SEQ ID NO:134);
Arg Leu Val Leu Val Leu Gly Trp (SEQ ID NO:135);
Trp Lys Trp Gly Ile Tyr Gly Trp (SEQ ID NO:136);
Ser Ser His Ala Ser Ala Gly Trp (SEQ ID NO:137);
Trp Gly Pro Trp Ser Phe Gly Trp (SEQ ID NO:138);
15 Ala Ile Pro Gly Lys Val Gly Trp (SEQ ID NO:139);
Arg Val His Asp Pro Ala Gly Trp (SEQ ID NO:140);
Arg Ser Val Ser Ser Phe Gly Trp (SEQ ID NO:141);
Leu Gly Thr Arg Lys Gly Gly Trp (SEQ ID NO:142);
Lys Asp Pro Leu Phe Asn Gly Trp (SEQ ID NO:143);
20 Leu Ser Gln His Thr Asn Gly Trp (SEQ ID NO:144);
Asn Arg Leu Leu Leu Thr Gly Trp (SEQ ID NO:145);
Tyr Pro Leu Trp Val Ile Gly Trp (SEQ ID NO:146);
Leu Leu Ile Ile Asp Arg Gly Trp (SEQ ID NO:147);
Arg Val Ile Ser Leu Gln Gly Trp (SEQ ID NO:148);
25 Glu Val Ser Arg Glu Asp Gly Trp (SEQ ID NO:149);
Ser Ile Leu Arg Ser Thr Gly Trp (SEQ ID NO:150);
Pro Gly Leu Val Trp Leu Gly Trp (SEQ ID NO:151);
Val Lys Lys Leu Tyr Ile Gly Trp (SEQ ID NO:152);
Asn Asn Arg Leu Leu Asp Gly Trp (SEQ ID NO:153);
30 Ser Lys Gly Arg Trp Gly Gly Trp (SEQ ID NO:154);
Ile Arg Pro Ser Gly Ile Gly Trp (SEQ ID NO:155);

Ala Ser Leu Cys Pro Thr Gly Trp (SEQ ID NO:156);
 Asp Val Pro Gly Leu Arg Gly Trp (SEQ ID NO:157);
 Arg His Arg Glu Val Gln Gly Trp (SEQ ID NO:158);
 Leu Ala Arg Lys Arg Ser Gly Trp (SEQ ID NO:159);
 5 Ser Val Leu Asp His Val Gly Trp (SEQ ID NO:160);
 Asn Leu Leu Arg Arg Ala Gly Trp (SEQ ID NO:161);
 Ser Gly Ile Ser Ala Trp Gly Trp (SEQ ID NO:162);
 Phe Tyr Phe Trp Val Arg Gly Trp (SEQ ID NO:163);
 Lys Leu Phe Leu Pro Leu Gly Trp (SEQ ID NO:164);
 10 Thr Pro Thr Leu Ser Asp Gly Trp (SEQ ID NO:165);
 Thr His Ser Leu Ile Leu Gly Trp (SEQ ID NO:166);
 Leu Leu Leu Leu Ser Arg Gly Trp (SEQ ID NO:167);
 Leu Leu Arg Val Arg Ser Gly Trp (SEQ ID NO:168);
 Glu Arg Arg ser Arg Gly Gly Trp (SEQ ID NO:169);
 15 Arg Met Leu Gln Leu Ala Gly Trp (SEQ ID NO:170);
~~Arg~~ Arg Gly Trp Ala Asn Ser Gly Trp (SEQ ID NO:171);
 Arg Pro Phe Tyr Ser Tyr Gly Trp (SEQ ID NO:172);
 Ser Ser Ser Trp Asn Ala Gly Trp (SEQ ID NO:173);
 Leu Gly His Leu Glu Glu Gly Trp (SEQ ID NO:174);
 20 Ser Ala Val Thr Asn Thr Gly Trp (SEQ ID NO:175);
 Leu Arg Arg Ala Ser Leu Trp (SEQ ID NO:176);
 Leu Arg Arg Trp Ser Leu Trp (SEQ ID NO:177);
 Lys Trp Val His Leu Phe Trp (SEQ ID NO:178);
 Asn Arg Leu Leu Leu Thr Trp (SEQ ID NO:179);
 25 Ala Arg Leu Leu Leu Thr Trp (SEQ ID NO:180);
 Asn Ala Leu Leu Leu Thr Trp (SEQ ID NO:181);
 Asn Arg Leu Ala Leu Thr Trp (SEQ ID NO:182);
 Asn Leu Leu Arg Leu Thr Trp (SEQ ID NO:183);
 Asn Arg Leu Trp Leu Thr Trp (SEQ ID NO:184); and
 30 Asn Arg Leu Leu Leu Ala Trp (SEQ ID NO:185).

[0066] Other heat shock protein binding domains useful in the practice of the present invention include Phe Tyr Gln Leu Ala Leu Thr Trp (SEQ ID NO:186), Phe Tyr Gln Leu

Ala Leu Thr Trp (SEQ ID NO:187), Arg Lys Leu Phe Phe Asn Leu Arg Trp (SEQ ID NO:188), Arg Lys Leu Phe Phe Asn Leu Arg Trp (SEQ ID NO:189), Lys Phe Glu Arg Gln Trp (SEQ ID NO:190), Asn Ile Val Arg Lys Lys Lys Trp (SEQ ID NO:191), and Arg Gly Tyr Val Tyr Gln Gly Leu Trp (SEQ ID NO:192).

- 5 [0067] Moreover, other heat shock protein binding domains include those described in WO9922761, and may have a terminal Trp residue added to achieve the purposes of the present invention. Xaa represents any amino acid.

- Tyr Thr Leu Val Gln Pro Leu Trp (SEQ ID NO:193);
Thr Pro Asp Ile Thr Pro Lys Trp (SEQ ID NO:194);
10 Thr Tyr Pro Asp Leu Arg Tyr Trp (SEQ ID NO:195);
Asp Arg Thr His Ala Thr Ser Trp (SEQ ID NO:196);
Met Ser Thr Thr Phe Tyr Ser Trp (SEQ ID NO:197);
Tyr Gln His Ala Val Gln Thr Trp (SEQ ID NO:198);
Phe Pro Phe Ser Ala Ser Thr Trp (SEQ ID NO:199);
15 Ser Ser Phe Pro Pro Leu Asp Trp (SEQ ID NO:200);
Met Ala Pro Ser Pro Pro His Trp (SEQ ID NO:201);
Ser Ser Phe Pro Asp Leu Leu Trp (SEQ ID NO:202);
His Ser Tyr Asn Arg Leu Pro Trp (SEQ ID NO:203);
His Leu Thr His Ser Gln Arg Trp (SEQ ID NO:204);
20 Gln Ala Ala Gln Ser Arg Ser Trp (SEQ ID NO:205);
Phe Ala Thr His His Ile Gly Trp (SEQ ID NO:206);
Ser Met Pro Glu Pro Leu Ile Trp (SEQ ID NO:207);
Ile Pro Arg Tyr His Leu Ile Trp (SEQ ID NO:208);
Ser Ala Pro His Met Thr Ser Trp (SEQ ID NO:209);
25 Lys Ala Pro Val Trp Ala Ser Trp (SEQ ID NO:210);
Leu Pro His Trp Leu Leu Ile Trp (SEQ ID NO:211);
Ala Ser Ala Gly Tyr Gln Ile Trp (SEQ ID NO:212);
Val Thr Pro Lys Thr Gly Ser Trp (SEQ ID NO:213);
Glu His Pro Met Pro Val Leu Trp (SEQ ID NO:214);
30 Val Ser Ser Phe Val Thr Ser Trp (SEQ ID NO:215);
Ser Thr His Phe Thr Trp Pro Trp (SEQ ID NO:216);

Gly Gln Trp Trp Ser Pro Asp Trp (SEQ ID NO:217);
 Gly Pro Pro His Gln Asp Ser Trp (SEQ ID NO:218);
 Asn Thr Leu Pro Ser Thr Ile Trp (SEQ ID NO:219);
 His Gln Pro Ser Arg Trp Val Trp (SEQ ID NO:220);
 5 Tyr Gly Asn Pro Leu Gln Pro Trp (SEQ ID NO:221);
 Phe His Trp Trp Trp Gln Pro Trp (SEQ ID NO:222);
 Ile Thr Leu Lys Tyr Pro Leu Trp (SEQ ID NO:223);
 Phe His Trp Pro Trp Leu Phe Trp (SEQ ID NO:224);
 Thr Ala Gln Asp Ser Thr Gly Trp (SEQ ID NO:225);
 10 Phe His Trp Trp Trp Gln Pro Trp (SEQ ID NO:226);
 Phe His Trp Trp Asp Trp Trp Trp (SEQ ID NO:227);
 Glu Pro Phe Phe Arg Met Gln Trp (SEQ ID NO:228);
 Thr Trp Trp Leu Asn Tyr Arg Trp (SEQ ID NO:229);
 Phe His Trp Trp Trp Gln Pro Trp (SEQ ID NO:230);
 15 Gln Pro Ser His Leu Arg Trp Trp (SEQ ID NO:231);
 Ser Pro Ala Ser Pro Val Tyr Trp (SEQ ID NO:232);
 Phe His Trp Trp Trp Gln Pro Trp (SEQ ID NO:233);
 His Pro Ser Asn Gln Ala Ser Trp (SEQ ID NO:234);
 Asn Ser Ala Pro Arg Pro Val Trp (SEQ ID NO:235);
 20 Gln Leu Trp Ser Ile Tyr Pro Trp (SEQ ID NO:236);
 Ser Trp Pro Phe Phe Asp Leu Trp (SEQ ID NO:237);
 Asp Thr Thr Leu Pro Leu His Trp (SEQ ID NO:238);
 Trp His Trp Gln Met Leu Trp Trp (SEQ ID NO:239);
 Asp Ser Phe Arg Thr Pro Val Trp (SEQ ID NO:240);
 25 Thr Ser Pro Leu Ser Leu Leu Trp (SEQ ID NO:241);
 Ala Tyr Asn Tyr Val Ser Asp Trp (SEQ ID NO:242);
 Arg Pro Leu His Asp Pro Met Trp (SEQ ID NO:243);
 Trp Pro Ser Thr Thr Leu Phe Trp (SEQ ID NO:244);
 Ala Thr Leu Glu Pro Val Arg Trp (SEQ ID NO:245);
 30 Ser Met Thr Val Leu Arg Pro Trp (SEQ ID NO:246);
 Gln Ile Gly Ala Pro Ser Trp Trp (SEQ ID NO:247);
 Ala Pro Asp Leu Tyr Val Pro Trp (SEQ ID NO:248);
 Arg Met Pro Pro Leu Leu Pro Trp (SEQ ID NO:249);

Ala Lys Ala Thr Pro Glu His Trp (SEQ ID NO:250);
 Thr Pro Pro Leu Arg Ile Asn Trp (SEQ ID NO:251);
 Leu Pro Ile His Ala Pro His Trp (SEQ ID NO:252);
 Asp Leu Asn Ala Tyr Thr His Trp (SEQ ID NO:253);
 5 Val Thr Leu Pro Asn Phe His Trp (SEQ ID NO:254);
 Asn Ser Arg Leu Pro Thr Leu Trp (SEQ ID NO:255);
 Tyr Pro His Pro Ser Arg Ser Trp (SEQ ID NO:256);
 Gly Thr Ala His Phe Met Tyr Trp (SEQ ID NO:257);
 Tyr Ser Leu Leu Pro Thr Arg Trp (SEQ ID NO:258);
 10 Leu Pro Arg Arg Thr Leu Leu Trp (SEQ ID NO:259);
 Thr Ser Thr Leu Leu Trp Lys Trp (SEQ ID NO:260);
 Thr Ser Asp Met Lys Pro His Trp (SEQ ID NO:261);
 Thr Ser Ser Tyr Leu Ala Leu Trp (SEQ ID NO:262);
 Asn Leu Tyr Gly Pro His Asp Trp (SEQ ID NO:263);
 15 Leu Glu Thr Tyr Thr Ala Ser Trp (SEQ ID NO:264);
 Ala Tyr Lys Ser Leu Thr Gln Trp (SEQ ID NO:265);
 Ser Thr Ser Val Tyr Ser Ser Trp (SEQ ID NO:266);
 Glu Gly Pro Leu Arg Ser Pro Trp (SEQ ID NO:267);
 Thr Thr Tyr His Ala Leu Gly Trp (SEQ ID NO:268);
 20 Val Ser Ile Gly His Pro Ser Trp (SEQ ID NO:269);
 Thr His Ser His Arg Pro Ser Trp (SEQ ID NO:270);
 Ile Thr Asn Pro Leu Thr Thr Trp (SEQ ID NO:271);
 Ser Ile Gln Ala His His Ser Trp (SEQ ID NO:272);
 Leu Asn Trp Pro Arg Val Leu Trp (SEQ ID NO:273);
 25 Tyr Tyr Tyr Ala Pro Pro Pro Trp (SEQ ID NO:274);
 Ser Leu Trp Thr Arg Leu Pro Trp (SEQ ID NO:275);
 Asn Val Tyr His Ser Ser Leu Trp (SEQ ID NO:276);
 Asn Ser Pro His Pro Pro Thr Trp (SEQ ID NO:277);
 Val Pro Ala Lys Pro Arg His Trp (SEQ ID NO:278);
 30 His Asn Leu His Pro Asn Arg Trp (SEQ ID NO:279);
 Tyr Thr Thr His Arg Trp Leu Trp (SEQ ID NO:280);
 Ala Val Thr Ala Ala Ile Val Trp (SEQ ID NO:281);
 Thr Leu Met His Asp Arg Val Trp (SEQ ID NO:282);

Thr Pro Leu Lys Val Pro Tyr Trp (SEQ ID NO:283);
 Phe Thr Asn Gln Gln Tyr His Trp (SEQ ID NO:284);
 Ser His Val Pro Ser Met Ala Trp (SEQ ID NO:285);
 His Thr Thr Val Tyr Gly Ala Trp (SEQ ID NO:286);
 5 Thr Glu Thr Pro Tyr Pro Thr Trp (SEQ ID NO:287);
 Leu Thr Thr Pro Phe Ser Ser Trp (SEQ ID NO:288);
 Gly Val Pro Leu Thr Met Asp Trp (SEQ ID NO:289);
 Lys Leu Pro Thr Val Leu Arg Trp (SEQ ID NO:290);
 Cys Arg Phe His Gly Asn Arg Trp (SEQ ID NO:291);
 10 Tyr Thr Arg Asp Phe Glu Ala Trp (SEQ ID NO:292);
 Ser Ser Ala Ala Gly Pro Arg Trp (SEQ ID NO:293);
 Ser Leu Ile Gln Tyr Ser Arg Trp (SEQ ID NO:294);
 Asp Ala Leu Met Trp Pro XAA Trp (SEQ ID NO:295);
 Ser Ser XAA Ser Leu Tyr Ile Trp (SEQ ID NO:296);
 15 Phe Asn Thr Ser Thr Arg Thr Trp (SEQ ID NO:297);
 Thr Val Gln His Val Ala Phe Trp (SEQ ID NO:298);
 Asp Tyr Ser Phe Pro Pro Leu Trp (SEQ ID NO:299);
 Val Gly Ser Met Glu Ser Leu Trp (SEQ ID NO:300);
 Phe XAA Pro Met Ile XAA Ser Trp (SEQ ID NO:301);
 20 Ala Pro Pro Arg Val Thr Met Trp (SEQ ID NO:302);
 Ile Ala Thr Lys Thr Pro Lys Trp (SEQ ID NO:303);
 Lys Pro Pro Leu Phe Gln Ile Trp (SEQ ID NO:304);
 Tyr His Thr Ala His Asn Met Trp (SEQ ID NO:305);
 Ser Tyr Ile Gln Ala Thr His Trp (SEQ ID NO:306);
 25 Ser Ser Phe Ala Thr Phe Leu Trp (SEQ ID NO:307);
 Thr Thr Pro Pro Asn Phe Ala Trp (SEQ ID NO:308);
 Ile Ser Leu Asp Pro Arg Met Trp (SEQ ID NO:309);
 Ser Leu Pro Leu Phe Gly Ala Trp (SEQ ID NO:310);
 Asn Leu Leu Lys Thr Thr Leu Trp (SEQ ID NO:311);
 30 Asp Gln Asn Leu Pro Arg Arg Trp (SEQ ID NO:312);
 Ser His Phe Glu Gln Leu Leu Trp (SEQ ID NO:313);
 Thr Pro Gln Leu His His Gly Trp (SEQ ID NO:314);
 Ala Pro Leu Asp Arg Ile Thr Trp (SEQ ID NO:315);

Phe Ala Pro Leu Ile Ala His Trp (SEQ ID NO:316);
 Ser Trp Ile Gln Thr Phe Met Trp (SEQ ID NO:317);
 Asn Thr Trp Pro His Met Tyr Trp (SEQ ID NO:318);
 Glu Pro Leu Pro Thr Thr Leu Trp (SEQ ID NO:319);
 5 His Gly Pro His Leu Phe Asn Trp (SEQ ID NO:320);
 Tyr Leu Asn Ser Thr Leu Ala Trp (SEQ ID NO:321);
 His Leu His Ser Pro Ser Gly Trp (SEQ ID NO:322);
 Thr Leu Pro His Arg Leu Asn Trp (SEQ ID NO:323);
 Ser Ser Pro Arg Glu Val His Trp (SEQ ID NO:324);
 10 Asn Gln Val Asp Thr Ala Arg Trp (SEQ ID NO:325);
 Tyr Pro Thr Pro Leu Leu Thr Trp (SEQ ID NO:326);
 His Pro Ala Ala Phe Pro Trp Trp (SEQ ID NO:327);
 Leu Leu Pro His Ser Ser Ala Trp (SEQ ID NO:328);
 Leu Glu Thr Tyr Thr Ala Ser Trp (SEQ ID NO:329);
 15 Lys Tyr Val Pro Leu Pro Pro Trp (SEQ ID NO:330);
 Ala Pro Leu Ala Leu His Ala Trp (SEQ ID NO:331);
 Tyr Glu Ser Leu Leu Thr Lys Trp (SEQ ID NO:332);
 Ser His Ala Ala Ser Gly Thr Trp (SEQ ID NO:333);
 Gly Leu Ala Thr Val Lys Ser Trp (SEQ ID NO:334);
 20 Gly Ala Thr Ser Phe Gly Leu Trp (SEQ ID NO:335);
 Lys Pro Pro Gly Pro Val Ser Trp (SEQ ID NO:336);
 Thr Leu Tyr Val Ser Gly Asn Trp (SEQ ID NO:337);
 His Ala Pro Phe Lys Ser Gln Trp (SEQ ID NO:338);
 Val Ala Phe Thr Arg Leu Pro Trp (SEQ ID NO:339);
 25 Leu Pro Thr Arg Thr Pro Ala Trp (SEQ ID NO:340);
 Ala Ser Phe Asp Leu Leu Ile Trp (SEQ ID NO:341);
 Arg Met Asn Thr Glu Pro Pro Trp (SEQ ID NO:342);
 Lys Met Thr Pro Leu Thr Thr Trp (SEQ ID NO:343);
 Ala Asn Ala Thr Pro Leu Leu Trp (SEQ ID NO:344);
 30 Thr Ile Trp Pro Pro Pro Val Trp (SEQ ID NO:345);
 Gln Thr Lys Val Met Thr Thr Trp (SEQ ID NO:346);
 Asn His Ala Val Phe Ala Ser Trp (SEQ ID NO:347);
 Leu His Ala Ala Xaa Thr Ser Trp (SEQ ID NO:348);

Thr Trp Gln Pro Tyr Phe His Trp (SEQ ID NO:349);
Ala Pro Leu Ala Leu His Ala Trp (SEQ ID NO:350);
Thr Ala His Asp Leu Thr Val Trp (SEQ ID NO:351);
Asn Met Thr Asn Met Leu Thr Trp (SEQ ID NO:352);
5 Gly Ser Gly Leu Ser Gln Asp Trp (SEQ ID NO:353);
Thr Pro Ile Lys Thr Ile Tyr Trp (SEQ ID NO:354);
Ser His Leu Tyr Arg Ser Ser Trp (SEQ ID NO:355); and
His Gly Gln Ala Trp Gln Phe Trp (SEQ ID NO:356).

10 [0068] The aforementioned heat shock protein binding domains are merely exemplary of various peptides, among peptide and non-peptide heat shock protein binding molecules, that may be used in the practice of the present invention.

15 [0069] The hybrid antigen of the invention incorporates one immunogenic domain and one heat shock protein-binding domain, optionally separated by a peptide linker. The hybrid antigen of the invention may be synthesized using chemical peptide synthesis methods or it can be synthesized by expression of a nucleic acid construct containing linked sequences encoding the antigenic and heat shock protein binding domains. One suitable technique utilizes initial separate PCR amplification reactions to produce separate DNA segments encoding the two domains, each with a linker segment attached to one end, followed by fusion of the two amplified products in a further PCR step. This technique is referred to as
20 linker tailing. Suitable restriction sites may also be engineered into regions of interest, after which restriction digestion and ligation is used to produce the desired hybrid antigen-encoding sequence.

25 [0070] As noted herein, the nucleic acid encoding a hybrid antigen of the invention is also suitable for therapeutic use by administration to the subject, where expression in vivo yields the hybrid antigen with the ability of inducing an immune response.

[0071] In addition to the studies on various heat shock protein binding domains and hybrid antigens containing them which are described above and in the Examples below, further comparative studies on heat shock protein binding domains, set forth using single-letter amino acid codes, HWDFAWPW (SEQ ID NO:358) and NLLRLTGW (SEQ ID
30 NO:359) were performed in vitro and in vivo, using the model epitope SIINFEKL (SEQ ID

NO:357) from ovalbumin. As mentioned above, antigenic peptides complexed to heat shock proteins (HSPs) are able to enter the endogenous antigen processing pathway and prime CD8+ cytotoxic T lymphocytes (CTLs). It was determined as mentioned above that the co-linear synthesis of a hybrid antigen containing a class I MHC binding epitope and an HSP70-binding sequence (HWDFAWPW) (SEQ ID NO:358) as well as other heat shock protein-binding sequences could render immunogenic otherwise poorly binding epitopes. This has been confirmed and extended by demonstrating that a higher-affinity HSP70-binding sequence (NLLRLTGW) (SEQ ID NO:359) can further enhance the immunogenicity of the bound class I epitope. A competition binding assay revealed a dissociation constant that was 15-fold lower for the H2-Kb ovalbumin SIINFEKL (SEQ ID NO:357) epitope in hybrid antigen SIINFEKLGSNLLRLTGW (SEQ ID NO:367) compared to hybrid antigen SIINFEKLGSGHWDFAWPW (SEQ ID NO:368), indicating a higher affinity of NLLRLTGW (SEQ ID NO:359) for HSP70. After confirming the ability of the HSP70-bound SIINFEKLGSNLLRLTGW (SEQ ID NO:367) peptide to be processed and presented by murine macrophages in vitro, in-vivo immunogenicity was assessed. The in-vivo comparative evaluation of HSP70:SIINFEKLGSNLLRLTGW (SEQ ID NO:367) vs. HSP70:SIINFEKLGSGHWDFAWPW (SEQ ID NO:368) complexes was performed by immunizing normal C57BL/6 mice, and the SIINFEKLGSNLLRLTGW (SEQ ID NO:367) complexes were found to induce better CD8+ T cell responses. In a related experiment, NLLRLTGW (SEQ ID NO:359) alone was found to prevent induction of an immune response by the otherwise-immunogenic HSP70:SIINFEKLGSGHWDFAWPW (SEQ ID NO:368) mixture, an indication that NLLRLTGW (SEQ ID NO:359) blocks complex formation by interacting with the same binding site on HSP70. It was hypothesized, for which Applicants have no duty to disclose nor are bound thereto, that the lower dissociation constant for epitopes containing NLLRLTGW (SEQ ID NO:359) would cause complexes made with HSP70 to be more efficient as immunizing agents. Indeed, it was found that animals immunized one time with a low dose of HSP70:SIINFEKLGSNLLRLTGW (SEQ ID NO:367) complexes induced peptide specific CD8+ T cells to secrete substantial levels of IFN-gamma in an ex vivo ELISPOT assay. Taken together, these data establish a positive correlation between the HSP70 binding affinity of the heat shock protein-binding domain-epitope hybrid antigen and immune responses, demonstrating that smaller amounts of defined epitopes can be used with a higher affinity heat shock protein binding domain such as but not limited to

NLLRLTGW (SEQ ID NO:359) to successfully immunize in vivo. Other heat shock protein binding domains terminated in a Trp residue were also found to have improved binding properties.

Heat Shock Proteins

5 [0072] The term "heat shock protein," as used herein, refers to any protein which exhibits increased expression in a cell when the cell is subjected to a stress. In preferred non-limiting embodiments, the heat shock protein is originally derived from a eukaryotic cell; in more preferred embodiments, the heat shock protein is originally derived from a mammalian cell. For example, but not by way of limitation, heat shock proteins which may
10 be used according to the invention include BiP (also referred to as grp78), hsp70, hsc70, gp96 (grp94), hsp60, hsp40, and hsp90, and members of the families thereof. Especially preferred heat shock proteins are BiP, gp96, and hsp70, as exemplified below. Most preferred is a member of the hsp70 family. Naturally occurring or recombinantly derived mutants of heat shock proteins may also be used according to the invention. For example,
15 but not by way of limitation, the present invention provides for the use of heat shock proteins mutated so as to facilitate their secretion from the cell (for example having mutation or deletion of an element which facilitates endoplasmic reticulum recapture, such as KDEL (SEQ ID NO:9) or its homologues; such mutants are described in PCT Application No. PCT/US96/13233 (WO 97/06685), which is incorporated herein by
20 reference).

[0073] For embodiments of the invention wherein heat shock protein and hybrid antigen are directly administered to the subject in the form of a protein/peptide complex, the heat shock protein may be prepared, using standard techniques, from natural sources, for example as described in Flynn et al., *Science* 245:385-390 (1989), or using recombinant
25 techniques such as expression of a heat shock encoding vector in a suitable host cell such as a bacterial, yeast or mammalian cell. If pre-loading of the heat shock protein with peptides from the host organism is a concern, the heat shock protein can be incubated with ATP and then repurified. Non-limiting examples of methods for preparing recombinant heat shock proteins are set forth below.

[0074] A nucleic acid encoding a heat shock protein may be operatively linked to elements necessary or desirable for expression and then used to express the desired heat shock protein as either a means to produce heat shock protein for use in a protein vaccine or, alternatively, in a nucleic acid vaccine. Elements necessary or desirable for expression include, but are not limited to, promoter/enhancer elements, transcriptional start and stop sequences, polyadenylation signals, translational start and stop sequences, ribosome binding sites, signal sequences and the like. For example, but not by way of limitation, genes for various heat shock proteins have been cloned and sequenced, including, but not limited to, gp96 (human: Genebank Accession No. X15187; Maki et al., *Proc. Natl. Acad. Sci. U.S.A.* 87:5658-5562 (1990); mouse: Genebank Accession No. M16370; Srivastava et al., *Proc. Natl. Acad. Sci. U.S.A.* 84:3807-3811 (1987)), BiP (mouse: Genebank Accession No. U16277; Haas et al., *Proc. Natl. Acad. Sci. U.S.A.* 85:2250-2254 (1988); human: Genebank Accession No. M19645; Ting et al., *DNA* 7:275-286 (1988)), hsp70 (mouse: Genebank Accession No. M35021; Hunt et al., *Gene* 87:199-204 (1990); human: Genebank Accession No. M24743; Hunt et al., *Proc. Natl. Acad. Sci. U.S.A.* 82:6455-6489 (1995)), and hsp40 (human: Genebank Accession No. D49547; Ohtsuka K., *Biochem. Biophys. Res. Commun.* 197:235-240 (1993)).

[0075] METHODS OF ADMINISTRATION

[0076] The hybrid antigens of the invention or complexes of hybrid antigens and heat shock proteins may be administered to a subject using either a peptide-based, protein-based or nucleic acid vaccine, so as to produce, in the subject, an amount of complex which is effective in inducing a therapeutic immune response in the subject.

[0077] The subject may be a human or nonhuman subject.

[0078] The term "therapeutic immune response," as used herein, refers to an increase in humoral and/or cellular immunity, as measured by standard techniques, which is directed toward the hybrid antigen. Preferably, but not by way of limitation, the induced level of humoral immunity directed toward hybrid antigen is at least four-fold, and preferably at least 16-fold greater than the levels of the humoral immunity directed toward the antigen prior to the administration of the compositions of this invention to the subject. The immune response may also be measured qualitatively, by means of a suitable *in vitro* or *in vivo*

assay, wherein an arrest in progression or a remission of neoplastic or infectious disease in the subject is considered to indicate the induction of a therapeutic immune response.

[0079] Specific amounts of heat shock protein/hybrid antigen administered may depend on numerous factors including the immunogenicity of the particular vaccine composition, the immunocompetence of the subject, the size of the subject and the route of administration. Determining a suitable amount of any given composition for administration is a matter of routine screening.

[0080] Furthermore, significant immunological efficacy was identified in studies in which the hybrid antigen was administered alone, i.e., without heat shock protein. While Applicants have no duty to disclose the theory by which the invention operates, and are not bound thereto, the results of these studies suggest that the hybrid antigens, upon injection into the subject, bind to endogenous heat shock proteins, and thus do not require the concomitant administration of heat shock protein for effectiveness. The present invention extends to such utilities of the hybrid antigens of the invention, and moreover, to concomitant therapies or treatments that increase endogenous heat shock protein levels systemically or at the intended site of administration of the hybrid antigens of the invention. Such concomitant therapies or treatments include but are not limited to local application of heat or local or systemic pharmaceutical agents that increase the expression of heat shock protein in the local tissue. Such agents and methods are known in the art.

[0081] Hybrid antigens that are administered in the absence of co-administration of a heat shock protein (i.e., administered not in a complex with a heat shock protein) that comprise at least one antigenic domain and at least one heat shock protein binding domain preferably comprise one of the heat shock protein binding domains described herein, and more preferably are hybrid antigens comprising the heat shock protein binding domains Asn Leu Leu Arg Leu Thr Gly Trp (SEQ ID NO:417), Phe Tyr Gln Leu Ala Leu Tyr Trp (SEQ ID NO:418), or Arg Lys Leu Phe Phe Asn Leu Arg Trp (SEQ ID NO:419). Other selections include those mentioned hereinabove.

[0082] In specific non-limiting embodiments of the invention, it may be desirable to include more than one species of heat shock protein, and/or more than one hybrid antigen,

in order to optimize the immune response. Such an approach may be particularly advantageous in the treatment of cancer or in the treatment of infections characterized by the rapid development of mutations that result in evasion of the immune response.

Moreover, a hybrid antigen of the invention may include more than one immunogenic
5 domain or more than one epitope.

[0083] Compositions comprising hybrid antigen/heat shock protein or hybrid antigen alone as set forth above are referred to herein as "vaccines." The term vaccine is used to indicate that the compositions of the invention may be used to induce a therapeutic immune response. A vaccine of the invention may comprise a hybrid antigen with a single antigenic
10 domain or epitope, or a hybrid antigen with a plurality of antigenic domains or epitopes. Further, a vaccine may comprise an admixture of hybrid antigens with single or pluralities of antigenic domains or epitopes, or any combination of the foregoing. As noted above, the hybrid antigens or admixtures thereof may be complexed with one or more heat shock proteins before administration, or may be administered without heat shock protein.

15 [0084] A vaccine composition comprising one or more hybrid antigens optionally complexed to one or more heat shock proteins in accordance with the invention may be administered cutaneously, subcutaneously, intradermally, intravenously, intramuscularly, parenterally, intrapulmonarily, intravaginally, intrarectally, nasally or topically. The vaccine composition may be delivered by injection, particle bombardment, orally or by aerosol.

20 [0085] Incubation of heat shock proteins in solution with the hybrid antigen is sufficient to achieve loading of the antigen onto the heat shock protein in most cases. It may be desirable in some cases, however, to add agents which can assist in the loading of the antigen.

[0086] Incubation with heating of the heat shock protein with the hybrid antigen will in
25 general lead to loading of the antigen onto the heat shock protein. In some cases, however, it may be desirable to add additional agents to assist in the loading. For example, hsp40 can facilitate loading of peptides onto hsp70. Minami et al., *J. Biol. Chem.* 271:19617-19624 (1996). Denaturants such as guanidinium HCl or urea can be employed to partially and reversibly destabilize the heat shock protein to make the peptide binding pocket more
30 accessible to the antigen.

[0087] Vaccine compositions in accordance with the invention may further include various additional materials, such as a pharmaceutically acceptable carrier. Suitable carriers include any of the standard pharmaceutically accepted carriers, such as phosphate buffered saline solution, water, emulsions such as an oil/water emulsion or a triglyceride emulsion, various types of wetting agents, tablets, coated tablets and capsules. An example of an acceptable triglyceride emulsion useful in intravenous and intraperitoneal administration of the compounds is the triglyceride emulsion commercially known as Intralipid®. Typically such carriers contain excipients such as starch, milk, sugar, certain types of clay, gelatin, stearic acid, talc, vegetable fats or oils, gums, glycols, or other known excipients. Such carriers may also include flavor and color additives or other ingredients.

[0088] In particular, a vaccine of the invention comprising a heat shock protein preferably also include adenosine diphosphate (ADP), to promote the association between the heat shock protein and the heat shock protein binding domain prior to the complex reaching its destination. Other compounds with similar capabilities may used, alone or in combination with ADP.

[0089] The vaccine composition of the invention may also include suitable diluents, preservatives, solubilizers, emulsifiers, adjuvants and/or carriers. Such compositions may be in the form of liquid or lyophilized or otherwise dried formulations and may include diluents of various buffer content (e.g., Tris-HCl, acetate, phosphate), pH and ionic strength, additives such as albumin or gelatin to prevent absorption to surfaces, detergents (e.g., Tween 20, Tween 80, Pluronic F68, bile acid salts), solubilizing agents (e.g. glycerol, polyethylene glycerol), anti-oxidants (e.g., ascorbic acid, sodium metabisulfite), preservatives (e.g., Thimerosal, benzyl alcohol, parabens), bulking substances or tonicity modifiers (e.g., lactose, mannitol), covalent attachment of polymers such as polyethylene glycol to the protein, complexing with metal ions, or incorporation of the material into or onto particulate preparations of polymeric compounds such as polylactic acid, polyglycolic acid, hydrogels, etc. or onto liposomes, microemulsions, micelles, unilamellar or multilamellar vesicles, erythrocyte ghosts, or spheroplasts. Such compositions will influence the physical state, solubility, stability, rate of *in vivo* release, and rate of *in vivo* clearance. The choice of compositions will depend on the physical and chemical properties of the vaccine. For example, a product derived from a membrane-bound form of a protein

may require a formulation containing detergent. Controlled or sustained release compositions include formulation in lipophilic depots (e.g. fatty acids, waxes, oils). Also comprehended by the invention are particulate compositions coated with polymers (e.g. poloxamers or poloxamines) and coupled to antibodies directed against tissue-specific receptors, ligands or antigens or coupled to ligands of tissue-specific receptors. Other embodiments of the compositions of the invention incorporate particulate forms protective coatings, protease inhibitors or permeation enhancers for various routes of administration, including intramuscular, parenteral, pulmonary, nasal and oral.

[0090] As an alternative to direct administration of the hybrid antigen optionally complexed with heat shock protein, one or more polynucleotide constructs may be administered which encode the hybrid antigen, optionally with heat shock protein, in expressible form. The expressible polynucleotide constructs are introduced into cells in the subject using *ex vivo* or *in vivo* methods. Suitable methods include injection directly into tissue and tumors, transfecting using liposomes (Fraley et al., *Nature* 370:111-117 (1980)), receptor-mediated endocytosis (Zatloukal et al., *Ann. NY Acad. Sci.* 660:136-153 (1992)), particle bombardment-mediated gene transfer (Eisenbraun et al., *DNA & Cell Biol.* 12:792-797 (1993)) and transfection using peptide presenting bacteriophage (Barry et al, *Nature Medicine* 2:299-305 (1996)). The polynucleotide vaccine may also be introduced into suitable cells *in vitro* which are then introduced into the subject.

[0091] To construct an expressible polynucleotide, a region encoding the heat shock protein and/or hybrid antigen is prepared as discussed above and inserted into a mammalian expression vector operatively linked to a suitable promoter such as the SV40 promoter, the cytomegalovirus (CMV) promoter or the Rous sarcoma virus (RSV) promoter. The resulting construct may then be used as a vaccine for genetic immunization. The nucleic acid polymer(s) could also be cloned into a viral vector. Suitable vectors include but are not limited to retroviral vectors, adenovirus vectors, vaccinia virus vectors, pox virus vectors and adenovirus-associated vectors. Specific vectors which are suitable for use in the present invention are pCDNA3 (InVitrogen), plasmid AH5 (which contains the SV40 origin and the adenovirus major late promoter), pRC/CMV (InVitrogen), pCMU II (Paabo et al., *EMBO J.* 5:1921-1927 (1986)), pZip-Neo SV (Cepko et al., *Cell* 37:1053-1062 (1984)) and pSR α (DNAX, Palo Alto, CA).

[0092] In the following examples, amino acids may be presented using their single-letter codes, as follows:

	[0093]	A alanine
	[0094]	C cysteine
5	[0095]	D aspartic acid
	[0096]	E glutamic acid
	[0097]	F phenylalanine
	[0098]	G glycine
	[0099]	H histidine
10	[00100]	I isoleucine
	[00101]	K lysine
	[00102]	L leucine
	[00103]	M methionine
	[00104]	N asparagine
15	[00105]	P proline
	[00106]	Q glutamine
	[00107]	R arginine
	[00108]	S serine
	[00109]	T threonine
20	[00110]	V valine
	[00111]	W tryptophan
	[00112]	Y tyrosine

EXAMPLE 1

[0100] A variety of hybrid antigens were prepared by solid-phase peptide synthesis,
25 each comprising a heat shock protein binding domain and a cancer antigen epitope or the
model epitope from ovalbumin, SIINFEEKL (SEQ ID NO:357) . The heat shock protein
binding domains used in these experiments were among the following: HWDFAWPW
(SEQ ID NO:358), NLLRLTGW (SEQ ID NO:359), FYQLALTW (SEQ ID NO:360) and
RKLFFNLRW (SEQ ID NO:361).

30 [0101] The cancer and model epitopes were among the following:

Source Protein	Source Tumor	Amino Acids	Trivial Name (Sequence)
Prostate Specific Membrane Antigen	Prostate cancer	771-779	PSMA P2 (ALFDIESKV) (SEQ ID NO:362)
Gp100	Melanoma	209-217	IMD (T210M) (IMDQVPFSV) (SEQ ID NO:363)
Tyrosinase	Melanoma	368-376	YMD (370D) (YMDGTMSQV) (SEQ ID NO:364)
Human Papillomavirus (HPV) Strain 16 E7	Cervical cancer	86-93	HPV16 E7 86-93 (TLGIVCPI) (SEQ ID NO:365)
HPV Strain 16 E7	Cervical cancer	11-20	HPV16 E711-20 (YMLDLQPETT) (SEQ ID NO:366)
Ovalbumin	Model Tumor Antigen	257-264	Ova (SIINFEKL) (SEQ ID NO:357)

[0102] Using standard solid phase peptide synthesis using F-moc chemistry, hybrid antigens comprising a heat shock protein binding domain, a cancer epitope, and a gly-ser-gly linker therebetween, were synthesized, in various orientations. Thus, the following

5 hybrid antigens were made:

Antigenic epitope	Heat shock protein-binding domain	Hybrid antigen sequence
ALFDIESKV (SEQ ID NO:362)	HWDFAWPW (SEQ ID NO:358)	ALFDIESKVgsgHWDFAWPW (SEQ ID NO:369)
IMDQVPFSV (SEQ ID NO:363)	HWDFAWPW (SEQ ID NO:358)	IMDQVPFSVgsgHWDFAWPW (SEQ ID NO:371)
IMDQVPFSV (SEQ ID NO:363)	NLLRLTGW (SEQ ID NO:359)	IMDQVPFSVgsgNLLRLTGW (SEQ ID NO:372)
YMDGTMSQV (SEQ ID NO:364)	HWDFAWPW (SEQ ID NO:358)	YMDGTMSQVgsgHWDFAWPW (SEQ ID NO:373)
YMDGTMSQV (SEQ ID NO:364)	HWDFAWPW (SEQ ID NO:358)	HWDFAWPWgsgYMDGTMSQV (SEQ ID NO:374)
YMDGTMSQV (SEQ ID NO:364)	NLLRLTGW (SEQ ID NO:359)	YMDGTMSQVgsgNLLRLTGW (SEQ ID NO:375)
TLGIVCPI (SEQ ID NO:365)	HWDFAWPW (SEQ ID NO:358)	TLGIVCPIgsgHWDFAWPW (SEQ ID NO:376)
TLGIVCPI (SEQ ID NO:365)	NLLRLTGW (SEQ ID NO:359)	TLGIVCPIgsgNLLRLTGW (SEQ ID NO:377)
YMLDLQPETT (SEQ ID NO:366)	HWDFAWPW (SEQ ID NO:358)	YMLDLQPETTgsgHWDFAWPW (SEQ ID NO:378)
SIINFEKL (SEQ ID NO:357)	HWDFAWPW (SEQ ID NO:358)	SIINFEKLgsgHWDFAWPW (SEQ ID NO:368)
SIINFEKL (SEQ ID NO:357)	HWDFAWPW (SEQ ID NO:358)	HWDFAWPWgsgSIINFEKL (SEQ ID NO:379)
SIINFEKL (SEQ ID NO:357)	NLLRLTGW (SEQ ID NO:359)	SIINFEKLgsgNLLRLTGW (SEQ ID NO:380)
SIINFEKL (SEQ ID NO:357)	FYQLALTW (SEQ ID NO:360)	SIINFEKLgsgFYQLALTW (SEQ ID NO:381)
SIINFEKL (SEQ ID NO:357)	RKLFFNLRW (SEQ ID NO:361)	SIINFEKLgsgRKLFFNLRW (SEQ ID NO:382)

EXAMPLE 2

[0103] Binding affinities between recombinant human or murine heat shock protein 70 (hsp70) and the various heat shock protein binding domains and antigenic peptides mentioned above, as well as between the hybrid antigens comprising an antigenic peptide and a heat shock protein binding domain described above, were determined by a binding inhibition assays (Hill plots) relative to the binding affinity of a reference, labeled hybrid antigen (tritiated ALFDIESKVGSGHWDFAWPW) (SEQ ID NO:369) to hsp70 as

determined by Scatchard analysis. Binding studies were performed in 39% PBS; 20 mM THAM, pH 8; 37 mM NaCl, 5 mM MgCl₂; and 1 mM ADP.

[0104] The affinities of hybrid antigens comprising the foregoing tumor antigenic peptides at the N-terminus (unless otherwise indicated), the indicated heat shock protein binding domain at the C-terminus, separated by a GSG linker, are set forth in the following table, values are expressed as Kd.

Peptide (epitope)	Heat shock protein-binding domain:				
	None	HWDFAWPW (SEQ ID NO:358)	NLLRLTGW (SEQ ID NO:359)	FYQLALTW (SEQ ID NO:360)	RKLFFNLRW (SEQ ID NO:361)
None		120 μ M	1.85 μ M	5.9 μ M	15 μ M
OVA	279 μ M	40 μ M	1.2 μ M	1.43 μ M	7.0 μ M
OVA (C-terminus)	279 μ M	4.3 μ M			
PSMA P1		53 Mm			
PSMA P2	145 μ M	22 μ M	1.3 μ M		
Gp100 ("IMD")	2565 μ M	155 μ M	3.1 μ M		
Tyrosinase ("YMD")	204 μ M	24 μ M	2.65 μ M		
Tyrosinase ("YMD") (C-terminus)	204 μ M	29 μ M			
HPV16 E7 (86-93)	187 μ M	9.1 μ M	5.2 μ M		
HPV16 E7 (11-20)	91 μ M	27 μ M			

EXAMPLE 3

[0105] Non-covalent complexes of recombinant human heat shock protein 70 and hybrid antigens of the invention were evaluated for biological activity *in vivo*. To evaluate the induction of an antigen-specific immune response, mice were immunized on day 0 with one of the following: (1) 100 μ g human hsp70; (2) a noncovalent complex of 100 μ g hsp70 and 15 μ g SIINFELK (SEQ ID NO:357) (OVA peptide); (3) a noncovalent complex of 100 μ g hsp70 and 15 μ g SIINFELKLGSGHWDFAWPW (SEQ ID NO:368) (N-terminal OVA

peptide, C-terminal heat shock protein binding domain HWDFAWPW (SEQ ID NO:358), GSG linker in between); and (4) a noncovalent complex of 15 µg hsp70 and 15 µg SIINFEKLGSGNLLRLTGW (SEQ ID NO:367) (N-terminal OVA peptide, C-terminal heat shock protein binding domain NLLRLTGW (SEQ ID NO:359), GSG linker in between).

5 [0106] On day 7, splenocytes were obtained from the immunized animals and were restimulated with peptide (SIINFEKL) (SEQ ID NO:357) *in vitro* for 5 days, after which the percent of γ -interferon-secreting CD8+ cells was determined. The results are shown in Figure 1.

[0107] Hsp70 alone showed a low level of response, which was not increased by the
10 addition of OVA peptide alone (complex of hsp70 and SIINFEKL (SEQ ID NO:357)). However, the complex of hsp70 and the hybrid antigen SIINFEKLGSGHWDFAWPW (SEQ ID NO:368) elicited an increased response. The complex comprising hsp70 and a hybrid antigen comprising OVA peptide and a higher affinity heat shock protein binding domain, NLLRLTGW (SEQ ID NO:359), gave about the same level of immune response,
15 but this was achieved using a 6.7-fold lower amount of hsp70 (15 µg).

EXAMPLE 4

[0108] Non-covalent complexes of recombinant human heat shock protein 70 and hybrid antigens of the invention were evaluated for biological activity in in-vitro models. An in-vitro model system was established in which murine peritoneal exudates cells were
20 exposed to a test compound of OVA peptide in the presence of B3Z cells, i.e., T-cell hybridomas that secrete IL-2 when presented with OVA peptide in the context of MHC Class I. Murine peritoneal macrophages were induced by an intraperitoneal injection of thioglycollate. Five days later, mice were sacrificed and peritoneal exudates cells were recovered by peritoneal lavage. Non-adherent cells were removed and then B3Z cells and
25 test compounds were added. The following were tested: 1- Hybrid Antigen "A" (10 pmol) (SIINFEKL—GSG—HWDFAWPW) (SEQ ID NO:368); 2- Noncovalent complex of Hsp70 (75 pmol) and Hybrid Antigen A (10 pmol); 3- Hybrid Antigen "B" (10 pmol) (SIINFEKL—GSG—NLLRLTGW) (SEQ ID NO:367); and 4- Noncovalent complex of Hsp70 (75 pmol) and Hybrid Antigen B (10 pmol).

[0109] Cell-free supernatants were harvested after 18h and tested in capture ELISAs for levels of IL-2. Both hybrid antigens in complexes with hsp70 presented antigen to the OVA-specific T-cell hybridomas, the results of which are set forth in Figure 2.

EXAMPLE 5

5 [0110] A Phase I/IIa clinical study in stage III and IV melanoma patients was conducted. Twenty-seven stage III/IV melanoma patients were divided into three groups and administered five doses of one of three formulations (low, medium and high dose) during a nineteen-week period. Nine patients received low doses, nine patients received medium doses, and nine patients received high doses. The components of the formulations
10 include two hybrid antigens mentioned above, each having a tumor antigenic domain (epitope) and an hsp70 binding domain, complexed with recombinant human hsp70, as follows:

Hybrid Antigen "I"

15 YMDGTMSQV—GSG—HWDFAWPW (SEQ ID NO:376) (Amino acids 368-378 of the melanoma tumor-associated antigen tyrosinase (YMDGTMSQV) (SEQ ID NO:364), GSG linker, Hsp70 binding domain HWDFAWPW) (SEQ ID NO:358)

Hybrid Antigen "II"

20 IMDQVPFSV—GSG—HWDFAWPW (SEQ ID NO:371) (Amino acids 209-217 of the melanoma tumor-associated antigen gp100 (IMDQVPFSV) (SEQ ID NO:363), GSG linker, Hsp70 binding domain HWDFAWPW) (SEQ ID NO:358)

[0111] In the low, medium, and high dose groups, either 1, 10 or 100 micrograms, respectively, of both of the foregoing hybrid antigens were formulated with 200 micrograms
25 of recombinant human hsp70, and administered to patients at weeks 0, 1, 2, 6, and 18. Bloods were drawn for immunological assays pre-immunization and at 8, 19 and 30 weeks. Fifteen patients' samples were evaluable for T-cell immunity, five patients in each dose group. A positive response was defined as a two-fold or greater increase in peptide-specific CD8+ T cells, measured by tetramer staining.

[0112] Overall, in 74% (20 of 27) of the patients, disease had not progressed at a median follow-up of 20 months. Of those 15 patients with evaluable blood samples, sixty percent (9 of 15) showed an increase in peptide-specific CD8+ T cells, and more positive T-cell responders were observed in the high-dose group. Only one (1/9) of these patients showed progression of disease. In contrast, disease progressed in three of the 6 patients who did not show a positive T-cell response.

EXAMPLE 6

[0113] Figure 3 shows various concentrations of SIINFEKL (SEQ ID NO:357) (Ova), SIINFEKLGSGHWDFAWPW (SEQ ID NO:368) or SIINFEKLGSGNLLRLTGW (SEQ ID NO:367) hybrid antigens titrated into binding reactions containing constant amounts of both HSP70 and a labeled reporter peptide of known affinity for HSP70. The abilities of these peptides to compete out the binding of the reporter were analyzed using a Hill plot and the IC50 of each determined as the point where the plot intersected the y-axis. The Kd of each peptide was then calculated from its experimentally determined IC50. There was a 15-fold increase in affinity of SIINFEKLGSGNLLRLTGW (SEQ ID NO:367) over SIINFEKLGSGHWDFAWPW (SEQ ID NO:368).

EXAMPLE 7

[0114] Peptides, alone or in a complex with HSP70, were added to adherent peritoneal exudate cells from thioglycollate induced mice. These cells were then cocultured with the B3Z T cell hybridoma, which produces IL-2 upon recognition of SIINFEKL (SEQ ID NO:357) in the context of H-2K^b. After an 18h incubation in serum free medium, supernatants were harvested and tested by ELISA. Data are shown in Figure 4a and 4b as the mean +/- S.D. Fig. 4a shows supernatant IL-2 quantities; Fig. 4b shows fold-induction of IL-2 over hybrid antigen alone.

EXAMPLE 8

[0115] C57BL/6 mice were immunized s.c. at the base of the tail with 2 mg hybrid antigen complexed with HSP or the appropriate controls. 7d later, mice were euthanized, CD8+ T cells were enriched from the spleens and put into an *ex vivo* ELISPOT to measure

IFN- γ production. Data are shown in Figure 5 as the mean \pm SE for \geq four experiments containing at least 3 mice per observation per experiment.

EXAMPLE 9

[0116] Mice were immunized s.c. at the base of the tail with the complexes listed above
5 – NLLRLTGW (SEQ ID NO:359) alone was added at a five-fold molar excess of the
SIINF EKLGSGHWDFAWPW (SEQ ID NO:358) dose. 7d later, spleens were harvested
and stimulated in vitro with SIINF EKL (SEQ ID NO:357) peptide. After 5d, peptide-
specific effector responses were measured in a ^{51}Cr release assay, results shown in Figure 6.
Values within bars represent the amount of epitope delivered. 100:1 E:T ratio shown.

EXAMPLE 10

[0117] Mice were immunized s.c. at the base of the tail with the indicated doses of
hybrid antigen with or without HSP70 or HSP70 alone. 7d later, spleens were harvested and
enriched for CD8+ T cells, which were put into an *ex vivo* IFN- γ ELISPOT assay. Data are
shown in Figure 7 as mean \pm standard error of \geq four experiments with at least three mice
15 per group.

[0118] The foregoing Examples show that a higher-affinity HSP70 binding sequence (in
these non-limiting examples, NLLRLTGW) (SEQ ID NO:359) can decrease the
dissociation constant of a hybrid antigen containing this sequence and a known class I MHC
antigenic epitope SIINF EKL (SEQ ID NO:357). The higher affinity of the heat shock
20 protein-binding domain-epitope hybrid antigen does not adversely affect the ability of the
immunogenic epitope to be processed and presented by MHC class I molecules. There is a
positive correlation between the HSP70 binding affinity of the heat shock protein-binding
domain-epitope hybrid antigen and CD8+ T cell immune responses elicited. Thus, smaller
amounts of defined epitopes can be used with a higher-affinity heat shock protein-binding
25 domain such as but not limited to NLLRLTGW (SEQ ID NO:359) to successfully
immunize *in vivo*.

EXAMPLE 11

[0119] Additional hybrid antigens comprising a human melanoma cancer epitope at the N-terminus, a Gly- Ser-Gly linker and the heat shock protein-binding domain Asn Leu Leu Arg Leu Thr Gly Trp were prepared by solid-phase peptide synthesis, and their affinities for hsp70 determined as above, compared with the melanoma epitope alone. The following table sets forth the various melanoma epitopes (names of proteins abbreviated, followed by the amino acids in the epitope, followed by any change to the native sequence in parentheses) and their affinities for hsp70 expressed in μM , either alone or in a hybrid antigen as mentioned above.

Cancer Epitope: amino acids	Epitope alone		Hybrid antigen comprising epitope	
	Epitope sequence	Affinity for hsp70 (μM)	Hybrid antigen sequence	Affinity for hsp70 (μM)
Gp100:209-217 (210M)	IMDQVPFSV (SEQ ID NO:363)	2566	IMDQVPFSVGSGNLLRLTGW (SEQ ID NO:372)	0.9
Tyrosinase: 368- 376 (370D)	YMDGTMSQV (SEQ ID NO:364)	209	YMDGTMSQVGSGNLLRLTGW (SEQ ID NO:375)	1.8
MelA/MART1: 26-35 (27L)	ELAGIGILTV (SEQ ID NO:389)	45	ELAGIGILTVGSGNLLRLTGW (SEQ ID NO:395)	0.6
NY-ESO-1: 157- 165 (165V)	SLLMWITQV (SEQ ID NO:390)	114	SLLMWITQVGSGNLLRLTGW (SEQ ID NO:396)	2.2
Trp-2: 180-188	SVYDFFVWL (SEQ ID NO:391)	81	SVYDFFVWLGSGNLLRLTGW (SEQ ID NO:397)	2.9
MAGE-10: 254- 262	GLYDGMEHL (SEQ ID NO:392)	48	GLYDGMEHLGSGNLLRLTGW (SEQ ID NO:398)	1.1
GP100: 280-288 (288V)	YLEPGPVTV (SEQ ID NO:393)	71	YLEPGPVTVGSGNLLRLTGW (SEQ ID NO:399)	2.0
SSX-2: 41-49	KASEKIFYV (SEQ ID NO:394)	57	KASEKIFYVGSGNLLRLTGW (SEQ ID NO:400)	1.4

EXAMPLE 12

[0120] In a similar fashion to melanoma antigens in the previous example, hybrid antigens were synthesized using various HLA A2 HIV epitopes. Affinities for hsp70 of the epitopes alone and in a hybrid antigen are shown in the following table.

HIV Epitope: amino acids	Epitope alone		Hybrid antigen comprising epitope	
	Epitope sequence	Affinity for hsp70 (μM)	Hybrid antigen sequence	Affinity for hsp70 (μM)
Nef: 190-198	ALKHRAYEL (SEQ ID NO:401)	97	ALKHRAYELGSGNLLRLTGW (SEQ ID NO:409)	1.1
Pol: 464-472	ILKEPVHGV (SEQ ID NO:402)	83	ILKEPVHGVGSGNLLRLTGW (SEQ ID NO:410)	1.2
Gag/p17: 77-85 (79F)	SLFNTVATL (SEQ ID NO:403)	35	SLFNTVATLGSGNLLRLTGW (SEQ ID NO:411)	1.9
Pol: 263-273	VLDVGDAYF SV (SEQ ID NO:404)	110	VLDVGDAYFSVGSGNLLRLTGW (SEQ ID NO:412)	2.0
Pol: 334-342	VIIYQYMDL (SEQ ID NO:405)	91	VIIYQYMDLGSGNLLRLTGW (SEQ ID NO:413)	1.7
Gag: 77-85	SLYNTVATL (SEQ ID NO:406)	85	SLYNTVATLGSGNLLRLTGW (SEQ ID NO:414)	1.8
Vpr: 59-67	AIIRILQQL (SEQ ID NO:407)	93	AIIRILQQLGSGNLLRLTGW (SEQ ID NO:415)	0.6
Nef: 190-198	AFHHVAREL (SEQ ID NO:408)	84	AFHHVARELGSGNLLRLTGW (SEQ ID NO:416)	2.1

EXAMPLE 13

[0121] For immunological studies in mice, a murine MHC H2-K(b) epitope from ovalbumin, SIINFEKL (SEQ ID NO:357) (amino acids 257-264), and a H2-K(b) peptide
5 from the nucleoprotein of vesicular stomatitis virus (VSV), RGYVYQGL (SEQ ID NO:370) (amino acids 52-59) were used for the preparation of hybrid antigens. The following table sets forth the sequences and the affinities for hsp70 of the epitopes alone and in hybrid antigens.

Mouse Epitope	Epitope alone		Hybrid antigen comprising epitope	
	Epitope sequence	Affinity for hsp70 (μM)	Hybrid antigen sequence	Affinity for hsp70 (μM)
Ovalbumin: amino acids 257-264	SIINFEKL (SEQ ID NO:357)	235	NLLRLTGWGSGSIINFEKL (SEQ ID NO:383)	1.6
VSV nucleoprotein: amino acids 52-59	RGYVYQGL (SEQ ID NO:370)	82	NLLRLTGWGSGRGYVYQGL (SEQ ID NO:386)	1.4

EXAMPLE 14

[0122] Mice were immunized s.c. at the base of the tail with hsp70 alone, hsp70 complexed with SIINFEKL (SEQ ID NO:357), and hybrid SIINFEKL (SEQ ID NO:357) peptide with or without HSP70. The doses were adjusted such that each immunization contained the same amount of SIINFEKL (SEQ ID NO:357), except for hsp70 alone. Seven days later, spleens were harvested and enriched for CD8+ T cells, which were put into an *ex vivo* IFN-γ ELISPOT assay. Responses after pulsing with SIINFEKL (SEQ ID NO:357) (“SIINFEKL”) were recorded in the following table, which includes the doses, and the number of spots (mean ± standard error) per 4 × 10⁵ CD8 T cells, of ≥ four experiments with at least three mice per group. Controls included medium alone (“medium control”), unpulsed T cells (“unpulsed control”), T cells pulsed with a non-immunized peptide derived from VSV, RGYVYQGL (SEQ ID NO:370) (“VSV control”), and exposure to concanavalin A as a positive control (“Con A positive control”).

[0123] In the same experiment, a ⁵¹Cr-release assay as described above was done using SIINFEKL -pulsed target cells. At an effector to target cell ratio of 200:1, the percent killing results obtained are shown in the far right column of the following table.

(200-10)

Immunogen	Number of Spots per 400,000 cells					CTL assay: % killing at 200:1 E/T
	SIINFEKL	Medium control	Unpulsed control	VSV control	Con A positive control	
4.4 μg Hsp70	0.00 ± 0.00	1.50 ±2.12	0.67 ±0.58	0.33 ±0.58	834 ±28.3	0%
4.4 μg Hsp70 + 0.9 μg SIINFEKL (SEQ ID NO:357)	33.7 ± 7.09	0.00 ±0.00	0.33 ±0.58	0.00 ±0.00	1000 ±33.7	19%
4.4 μg Hsp70 + 2.0 μg	80.0	0.00	1.50	1.50	1170	38%

NLLRLTGWGSGSIINFEKL (SEQ ID NO:383)	± 17.0	±0.00	±0.71	±0.71	±56.5	
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EXAMPLE 15

[0124] An experiment similar to that described above was carried out, which also included hybrid antigen without hsp70.

5 (200-11)

Immunogen	Number of Spots per 4 x 10 ⁵ CD8 T cells				
	SIINFEKL	Medium control	Unpulsed control	VSV control	Con A Positive control
4.4 µg Hsp70	0.33 ± 0.58	1.00 ± 1.73	1.67 ± 1.15	4.00 ± 1.00	965 ± 62.6
4.4 µg Hsp70 + 0.9 µg SIINFEKL (SEQ ID NO:357)	1.67 ± 0.58	1.00 ± 1.00	2.00 ± 0.00	2.67 ± 2.08	591 ± 48.1
4.4 µg Hsp70 + 2.0 µg NLLRLTGWGSGSIINFEKL (SEQ ID NO:383)	12.0 ± 5.2	2.67 ± 0.58	1.67 ± 1.15	2.00 ± 2.65	748 ± 58.6

EXAMPLE 16

[0125] A further experiment was carried out similar to that described above.

10 (200-12)

Immunogen	Number of spots per 300,000 CD8 T cells					CTL assay: % killing at 200:1 E/T
	SIINFEKL	Medium control	Unpulsed control	VSV control	Con A positive control	
4.4 µg Hsp70	0.67 ± 0.58	0.00 ± 0.00	0.50 ± 0.71	1.00 ± 1.41	552 ± 24.0	8.45 ± 41.3
4.4 µg Hsp70 + 0.9 µg SIINFEKL (SEQ ID NO:357)	3.33 ± 2.52	0.00 ± 0.00	0.33 ± 0.58	0.33 ± 0.58	450 ± 69.0	43.0 ± 21.2
4.4 µg Hsp70 + 2.00 µg NLLRLTGWGSG-SIINFEKL (SEQ ID NO:383)	134 ± 4.16	1.33 ± 1.53	0.67 ± 1.15	1.00 ± 1.00	865 ± 93.0	31.9 ± 5.41

[0126] The present invention is not to be limited in scope by the specific embodiments describe herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and the accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

[0127] Various publications are cited herein, the contents of which are hereby incorporated by reference in their entireties.